PROGRESS IN BIOPHYSICS
AND BIOPHYSICAL CHEMISTRY
4

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EXTRACT FROM PREFACE TO VOLUME 1

THE editors have had some difficulty in deciding what is the proper field to be covered by reviews of recent progress in biophysics. Excluding biochemistry on the one hand and physiology on the other, there lies between a vast and rather amorphous field of study of which the frontiers and lines of demarcation are anything but well defined. On the one hand there is the application of the methods of physics to the study of the characteristic molecules of life—proteins, nucleoproteins, lipoproteins and other complex molecules of many and mostly unexplored varieties, a study which should perhaps be strictly regarded as an aspect of biophysical chemistry—hence the sub-title of the book. On the other hand there is the application of physical methods and instruments to the study and elucidation of living structures such as nerve and muscle fibres, a field in which modern refinements offer the greatest promise of progress in the future; progress which will perhaps bring its subjects into the first category. Both these fields of work are represented in the present volume and the editors have endeavoured to maintain a reasonable balance between them.

It has been our aim to obtain readable and interesting reviews of recent progress in selected subjects. Our contributors have been asked not merely to compile a précis of recent papers, or annotated lists of references, but to write critical reviews in which they discuss the subjects from their own point of view; which may be read with profit by many who are not experts and which will provide scientists with a general survey of recent work and ideas.

Since it is impossible in a single volume to deal at length with more than a few topics, it is intended that this volume will be followed by others at regular intervals. In time a more complete coverage of all the varied aspects of this rapidly developing branch of science will thus be built up.

May, 1950

J. A. V. BUTLER
J. T. RANDALL

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1

POLYELECTROLYTE GELS

A. Katchalsky

I. Introduction

The concept of polyelectrolytes—high polymer molecules carrying repeating ionizable groups—permits a new classification of the biocolloids and synthetic polymers according to their electrochemical properties. This classification has also the advantage of allowing the isolation of polyelectrolyte gels out of the enormous wealth of different gel structures encountered in the living organism and in technological applications.

Polyelectrolyte gels, which are the subject of this review, are homogeneous solids in the rheological sense,[1] comprising at least two components, one of which is a network of ionizable macromolecules. According to the nature of the ionizable groups the gels may be of three major types: polyacid, polybase, or polyampholyte. This classification applies to the biogels as well as to the other polyelectrolyte gels. Among the biogels, the most important group is the protein gels, which belong generically to the polyampholyte type. The other large group is the polyacids, mainly of the polysaccharide structure. To this group belong the polyuronic acid gels such as the pectic and pectinic acids—all of them composed of galacturonic acid monomers[2]—the alginic-acid gels built of repeating units of mannuronic acid,[3] the bacterial polyuronic-acid gels of immuno-chemical importance,[4] as well as the polysulphonic acids such as chondroitin and mucoitin sulphate.[3] The classical gel-forming substance, agar-agar, a polysaccharide carrying one sulphate group every tenth monomer, also belongs to this group.[5]

Another important group from the biological point of view comprises the gels resulting from the interaction of two or more polyelectrolytes. Prominent cases are the gel structures of the chromosomes, resulting from the interactions of nucleic acid with protamines and histones.[6] The gels of the connective tissues, produced by the interaction of procolagen with hyaluronic, chondroitin sulphuric, and mucoitin sulphuric acid,[7] and the recently-discovered constituents of the muscle derived from the interaction of actin and myosin[8] belong to this class of complex gels. The cytoplasm is apparently a loose gel network derived from the interaction of proteins with nucleic acids, lipoids and other constituents of the cell.[9], [10], [11] The polyelectrolyte biogels, and especially those resulting from the interaction of two or more components, present difficulties for physico-chemical investigation

because of the complexity both of the component macro-molecules and of the network. This group will not be followed up in this review and more attention will be devoted to the simpler biogels and to gels of synthetic polyelectrolytes, which permit a clearer and quantitative evaluation of the gel behaviour.

Among the synthetic polyelectrolyte gels may be mentioned the acidic derivatives of cellulose such as the xanthogenates,[12], [3b] carboxymethyl cellulose gels,[13] and the numerous resin ion exchangers.[14] The ion exchangers include the whole spectrum of acidic groups, from the weak aromatic hydroxyls[15] via carboxylic groups of different strength[16] to the strong polysulphonic acids,[17] as well as a variety of polybases ranging from imines to strong tertiary amines.[18]

Of special interest for theoretical consideration are the slightly cross-linked polyacrylic acids, polymethacrylic acids, and sulphonated polystyrenes, which give highly swollen dilute gels,[19] and the polyvinylphosphates, which have some of the characteristics of biogels.[20] Some interesting development has taken place recently in the field of synthetic polyampholyte gel-forming substances. JONES[21] has obtained polymers of acrylic acid amides of dibasic amino acids which give polyampholyte gels resembling gelatin.

No attempt is being made here to cover all the biological, physicochemical and technological aspects of the large group of polyelectrolyte gels. Each type of polyelectrolyte gel deserves a whole treatise and many of them have been described in special monographs. Our aim is to elucidate some of the common and fundamental principles that can be derived from the combined theories of polyelectrolytes and of network statistics. It is felt that a new approach, which utilizes the theoretical knowledge of both the polymer and electrolyte properties of polyelectrolytes, may provide a clearer interpretation of many known facts in the study of biogels as well as in the study of the synthetic ion exchangers. The task of the reviewer is made easier by several recent general reviews of gels, which contain valuable summaries of material pertaining to polyelectrolyte gels. The most comprehensive review is that of P. H. HERMANS,[22] while other authors have dealt with more restricted topics related to our subject. Pertinent are the review of FERRY[23] on protein gels, the reviews of BOYD, of WALTON, of BAUMAN, of MYERS, and of KUNIN and MYERS[24] on synthetic ion exchangers, and the reviews of DOTY, and of EISENBERG and FUOSS on polyelectrolytes.[25]

II. STRUCTURAL PRINCIPLES

Almost a hundred years ago, VON NAEGELI[26] realized that a necessary condition for the formation of a gel is the existence of a cross-linked network. The cross-linking may take place by covalent bonds, by van der Waals junctions, or by electrostatic links. The network may grow

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from polyfunctional monomers during polymerization, as with the synthetic tridimensional polymers, or may result from the junction of polymeric chains, as is usual with biogels.

In order for a gel to be formed, the probability of network growth must pass a certain critical value. When the cross-linking reaction has advanced to the stage at which the probability reaches this critical point—the gel point—an incipient network of negligible amount is formed, an abrupt increase in viscosity occurs, and the first indications of mechanical rigidity appear.[27] A thorough theoretical treatment of the probability of sol-gel transformation has been given by FLORY[28] and has been extended by STOCKMAYER[29] for the cross-linking of linear macromolecules of arbitrary initial size distribution. The theory leads to the conclusion that in the case of long-chain polymers the minimal concentration of cross-links required for the initial gel formation is

$$m_{\mathrm{cl}} = \frac{C}{2M_w} \qquad \ldots . (1)$$

where m_{cl} is the cross-link concentration in mol/litre, C is the concentration of polymer in grams/litre, and M_w is its weight-average molecular weight. In order to reach this critical cross-link concentration in solutions of polymers, the solubility should be reduced by alteration of the temperature, by addition of a non-solvent, or by addition of chemical reagents. The formation of polyelectrolyte gels is, in addition, strongly dependent on electrochemical factors which govern the intermolecular electrostatic repulsions and attractions. This topic will be discussed in more detail in part 3 of this section.

In the following paragraphs we shall deal with the structural principles governing gel behaviour as derived from the nature of network cross-links. The intimate organization of the network chains which leads to partial crystallization and to related optical phenomena has been summarized recently in a full and lucid fashion by P. H. HERMANS.[22] The reader is referred for details to his review. The X-ray and optical data on polyelectrolyte gels will be mentioned here only for the interpretation of other phenomena.

1. *Covalent cross-linkage*

Cross-linking of the gel network by primary chemical bonds is of major importance in technology and occurs frequently in biogels. The main covalent bonds of the protein chains in biogels are the S-S bonds,[30] amide bridges between the amino and carboxylic groups situated on neighbour chains,[31] and, apparently, ester and ether links. In protein technology cross-links are introduced in order to endow the gels with lower water absorption and higher chemical resistance. Well

known is the covalent cross-linking of collagen fibres in the process of tanning by different reagents and especially by aldehydes and quinones, as well as by carbon disulphide and ketene.[32] The bonding of collagen by formalin has been extensively studied and it seems that the junction consists of methylene bridges which join the ε-amino groups of lysine residues with the imino groups of peptide bonds;[33] of special interest is the formalin cross-linking of toxins which transforms them into toxoids, of great serological value.[34] During the last few years methods have been derived for cross-linking polyuronic acids also, by primary bonds, so as to give gels of limited swelling. DEUEL[35] reacted pectic and alginic acids with epoxides to give hydroxy esters, which are readily joined by formaldehyde according to the following scheme:

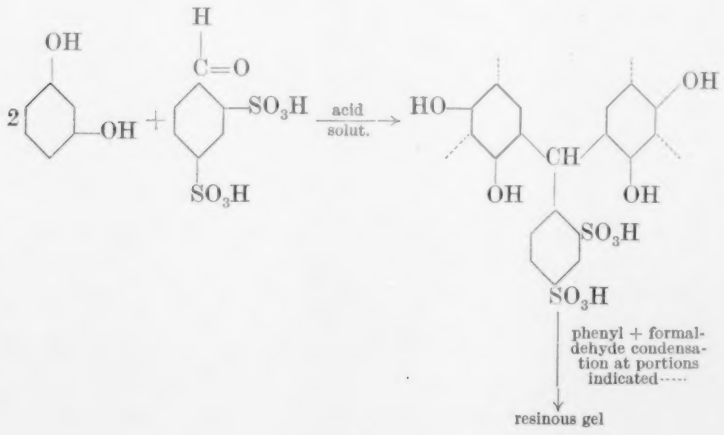
$$\mathrm{R{-}COOH} + \underset{\mathrm{O}}{\mathrm{CH_2{-}CH_2}} \rightarrow \mathrm{R{-}COOCH_2{-}CH_2OH}$$

$$2\,\mathrm{RCOOCH_2CH_2OH} + \mathrm{CH_2O} \rightarrow \mathrm{RCOOCH_2{-}CH_2{-}O{-}CH_2{-}OCH_2{-}CH_2{-}OOCR}.$$

Another linking agent is mustard gas, which gives sulphur bridges according to the following equation:

$$2\,\mathrm{RCOONa} + (\mathrm{Cl{-}CH_2{-}CH_2})_2\mathrm{S} \rightarrow \mathrm{RCOOCH_2CH_2{-}S{-}CH_2CH_2OOCR} + 2\mathrm{NaCl}.$$

The main cross-linking procedure in the production of ion exchangers is that used with the thermosetting plastics. The common syntheses are the poly-condensations of aromatic rings with formalin, e.g. the production of sulphonic cross-linked polyelectrolytes.[36]



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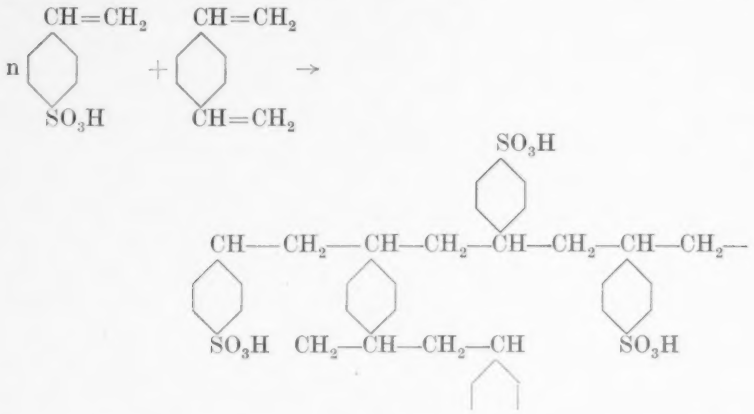
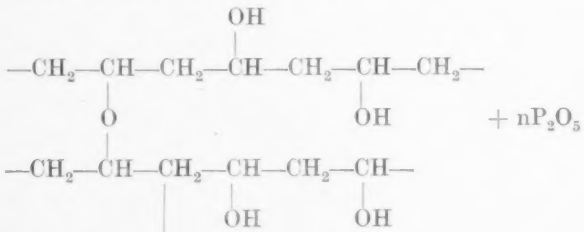
or copolymerization with divinyl benzene

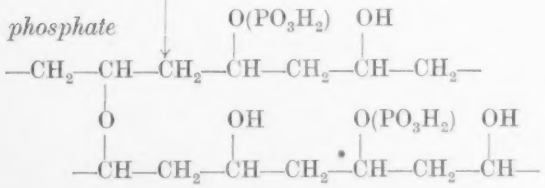


W. KUHN and HARGITAY[19d] have brought about the branching of polyacrylic acids by esterification with glycerol and polyvinyl alcohol. Cross-linked polyelectrolyte gels may also be obtained by the introduction of ionizable groups into a non-electrolytic tridimensional polymer. Thus contractile polyvinyl phosphate gels have been obtained by phosphorylation of polyvinyl alcohol cross-linked by ether groups.[19]

Cross-linked polyvinyl alcohol



Polyvinyl phosphate



Important from the technological point of view is the introduction of sulphonic groups[37] and quaternary ammonium groups[38] into cross-linked polystyrene.

The stability of the primary cross-links makes the network structure robust and almost independent of the nature of the imbibing medium

and of inner stresses. The network is not torn apart by absorbed solvent and the swelling of these gels does not lead to ultimate dissolution of the network. Swelling, even in the best solvents, is always limited and reaches a state of "equilibrium swelling". The theory of this limiting case has been extensively treated in numerous publications dealing with non-polar polymers.[39] This theory may also be applied to ionized polyelectrolyte gels, as will be shown in the next section.

For the theoretical description of the network we may assign definite molecular weights and correspondingly definite degrees of polymerization to the chains extending between neighbouring cross-linking junctions. In swollen gels, these networks chains are regarded as free units to which mixing entropy and elastic properties may be ascribed. The equation of FLORY and REHNER[40] as corrected recently by FLORY,[41] correlates explicitly the mean degree of polymerization Z of the "chains" of non-polar gels with the swelling volume V at equilibrium swelling and with μ'—the polymer-solvent interaction constant of HUGGINS.[42] The approximate equation which is used for the evaluation of Z is:

$$Z = \frac{V^{5/3} - \frac{1}{2}V}{\frac{1}{2} - \mu'} \qquad \ldots.(2)$$

The number of cross-links is usually unknown and may not readily be predicted even in synthetic gels where the amount of cross-linking agent is regulated by the experimenter. The usual procedure for the determination of Z is to measure the volume of the gel at equilibrium in a solvent of known μ'. Z is then evaluated from equation (2). The situation is even more complicated in polyelectrolyte gels, but careful chemical transformation of the polyelectrolyte gel into a non-polar gel allows FLORY'S equation to be used for the evaluation of Z.

In the case of polymethacrylic acid cross-linked by divinyl benzene[43] the following procedure was adopted: Dry, purified, cross-linked polymer was suspended in benzene containing an excess of diazomethane. The reaction mixture was left at room temperature till evolution of N_2 ceased. The resultant polyester was washed thoroughly with benzene and dried in vacuum. It was finally swollen to equilibrium in chloroform ($\mu' = 0{\cdot}32$)[44] and Z was calculated from the volume at equilibrium by equation (2).

2. *Van der Waals junctions*

Network formation—The formation of polyelectrolyte biogels by van der Waals forces, including hydrogen bonds, is by now well established. Recent investigations shed new light on the course of association of the polymeric molecules into networks held together by van der Waals forces.

Of special interest are the first steps by which native globular proteins transform into long chains capable of gel formation before irreversible denaturation occurs. WAUGH[45] observed that controlled

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heating of insulin at low *p*H causes the formation of long molecular fibrils which may be decomposed by alkali into insulin, identical with the original as regards crystalline structure and cataphoretic and ultracentrifugal behaviour.[46] FERRY[47] found that a similar fibrillation is the first step in the transformation of fibrinogen into fibrin during blood clotting. In the last few years several French investigators have proved that the same mechanism of necklace junction by van der Waals forces into longer fibrils is active in the gelation of blood proteins, gelatin, and casein.[48], [49], [50], [51] By measurements of double refraction of flow BARBU and JOLY[52] demonstrated the growth of the

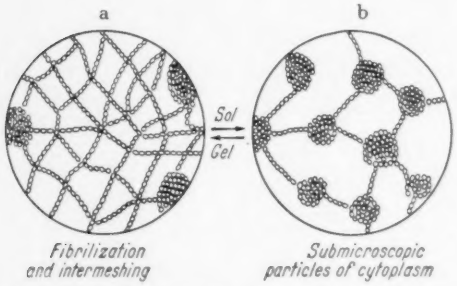

Fig. 1. Hypothetical ultra-structure of cytoplasm and its sol-gel transformation. The figure gives only the protein molecules although other substances, including nucleic acids and phospholipids, are associated with cytoplasmic proteins and particulates (from KAPAC, M. J.; *Ann. N.Y. Acad. Sci.* 51 (1951) 1542)

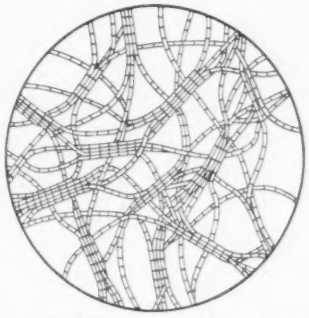

Fig. 2. Schematic representation of a well-developed gel containing crystalline regions of cross-linking (from HERMANN, K. and GERNGROSS, O.; *Kautschuk* 8 (1932) 181)

necklace fibrils on heating the serum proteins to about 80°C at low *p*H's and their reversible change to the native proteins on cooling to room temperature. The lability of the cross-links was demonstrated by measurements of streaming birefringence at high velocity gradients sufficient to decrease the length of the fibrils. On the basis of biological observations, KAPAC[53] came to the conclusion that the mechanism of the cytoplasmatic sol-gel transformation accompanying the formation of the spindle in cell division, as well as the irritation and cellular movement, is of the same type (Fig. 1).

After the first van der Waals links have been formed, a second process of slow crystallization takes place. During this process, which may last for days and sometimes weeks,[54] the classical network picture shown for gelatin in Fig. 2, is developed.

Stability of cross-links—Many physical properties of polyelectrolyte gels demonstrate the lability and reversibility of the van der Waals linkages. Already in 1904 LEICK[55] found that the modulus of rigidity of gelatin gels increases with the square of gelatin concentration.

LEICK's observations were confirmed by FERRY,[56] who extended the measurements to fractionated gelatins of known molecular weights in a range of concentrations from 0–5 per cent and temperatures from 0–30°C. FERRY inferred from his measurements that the number of cross-links increases with the square of concentration. According to the theory of rubberlike elasticity[57] the rigidity modulus increases linearly with the number of cross-links and thus LEICK's observation may be satisfactorily interpreted.

The reversible sol-gel transformation in the classical gel-forming substances, such as gelatin and agar, occurs at a definite transition temperature which may be regarded as the melting point of the gel. This

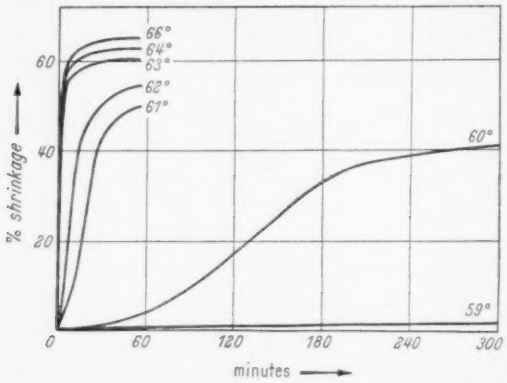

Fig. 3. Shrinkage of collagen fibres of the skin at different temperatures as a function of time (from GRASSMANN, W.; *Kolloid* Z. 77 (1936) 205)

point may be approached from the sol by a lowering of the temperature and then it is known as the setting point. Usually the melting point is defined with better precision than the setting point. It was found[23] that for gelatin gels of concentrations higher than 2 per cent, the melting point increases linearly with concentration. In the investigation of collagen and dermis it was observed that heating these substances in water to temperatures between 44–66°C results in rapid shrinkage.[58] The phenomenon is clearly shown in the experiments of GRASSMANN[59] represented in Fig. 3. At the same temperature at which the gels shrink, the elasticity of the network increases abruptly and the hard collagen becomes stretchable in a rubberlike fashion (Fig. 4).

It was found that these changes in the mechanical behaviour are not accompanied by any definite chemical change: no new reactive groups are liberated and the acid-base capacity remains the same.[59] On the other hand the crystalline x-ray picture of collagen becomes diffuse, resembling that of gelatin.[60] According to LLOYD,[61] the van der Waals forces joining the crystalline regions are broken at the shrinkage

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temperature and randomly-kinked chains are liberated. The thermal motion brings the chains into the more probable state, i.e. the contracted one, and this chain contraction explains the overall shrinkage of the gel. According to this view the shrinkage temperature is identical with the melting temperature of the gel. Similarly, the 17–25 Kcal per kg. absorbed during the shrinkage is interpreted as the heat of melting. Cooling the collagen to its initial temperature does not restore the original order; the X-ray picture therefore is not identical with that of the native collagen and the heat liberated on cooling is only 12–13 Kcal. If the relative positions of the molecular fibres are fixed by mild treatment with formalin[62] the reversibility of melting is increased. Interesting

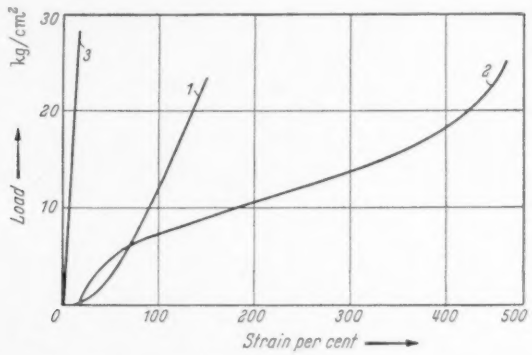

Fig. 4. Stress-strain relations in: (1) Collagen above its shrinking point, (2) slightly vulcanized rubber, (3) native collagen (from WÖHLISCH, E.; *Z. Biol.* 85 (1927) 325)

is the observation of WÖHLISCH[63] that the shrinkage temperature of stretched collagen increases with external stress. In the stretched fibres the relative positions of the cross-linking points are more rigidly fixed and higher thermal energies are required to break the junctions and disorient the molecular chains.

FLORY[64] has recently developed a theory of crystallization for pure and swollen polymers. FLORY's equation for the melting point T_m of a polymer containing crystallizable groups when mixed with a solvent is

$$\frac{1}{T_m} - \frac{1}{T_m^\circ} = \frac{R}{h_u}\{-\ln X_a - \mu'(1 - V_a)^2\} \qquad \ldots.(3)$$

where T_m° is the melting point of a pure polymer of infinite length, h_u is the heat of fusion, X_a and V_a are the mole fraction and volume fraction, respectively, of the crystallizing structural unit in the polymer, and μ' is the HUGGINS solvent-polymer interaction constant. This melting theory has not yet been applied to the complicated biogels or to

polyelectrolyte gels. It seems, however, that an extension of this theory would lead to important developments in the thermodynamic evaluation of the crystallization parameters.

Nature of the bonding groups—The nature of the van der Waals bonding groups has been extensively investigated by LLOYD and her collaborators.[65] By a study of solvents which cause radial swelling, axial shrinkage and solution of silk fibroin, collagen, and elastin, it was possible to identify the lateral bonds which hold the molecular chains together. Organic solvents which rupture only some of the bonds allow the molecules to shorten with corresponding gain in entropy. These solvents produce only axial shrinkage accompanied by rubberlike extensibility, while solvents which break all types of lateral bonds cause solution of the fibres. The hydrogen bonds of the carbamino groups are broken by *m*-cresol, formamide, formic acid, lactic acid, and thioglycolic acid, which bring the protein gels into solution. Treatment of collagen with solutions of urea breaks the hydrogen bonds and brings the melting point to room temperature, a depression of about 40°C.[66] A similar investigation on the hydrogen-bonding in actomyosin gel has been carried out by BOTTS,[67] who found that the most powerful dissolving agents, which easily rupture the hydrogen bonds, are diacetyl, pyridine, picoline, piperidine, and, as usual, urea solutions.

The study of the van der Waals interactions is complicated by the coulombic forces which operate in all the gels under consideration. In the next paragraph we shall therefore refer often to the combined action of van der Waals and electrostatic forces.

3. *Bonding by electrostatic forces*

The classical example of electrovalent bonding in polyelectrolyte gels is provided by the proteins at their isoelectric points. It is plausible to expect attractive forces to operate in the gel formation of proteins when the molecules carry approximately equal numbers of positive and negative charges. In dilute solutions these attractive forces will act only *intra*molecularly and will determine to a large extent the shape of the protein molecules. In concentrated solutions on the other hand *inter*molecular attractions will develop which will favour electrovalent junction between the macromolecules and will contribute to the formation of protein gels. Despite the dissociative action of water, due to its high dielectric constant, the electrostatic fields of macromolecules are sufficiently powerful to make the cross-linking by electrovalent bonds of major importance in polyelectrolyte gels. The contribution of the charged groups to the stabilization of the network is determined not only by their number and valency but also to a large extent by their spatial arrangement in the chain units and in the network. It should be clearly borne in mind that equality in number of positive and negative

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charges is not sufficient to produce effective bonding. Thus in randomly-kinked fibrillar molecules of the gelatin type the overall electrostatic attractions at the isoelectric point will be negligible as compared with other forces. On the other hand, in highly organized collagen, in which the natural arrangement of the network elements provides for close proximity of positive and negative groups, the electrostatic attraction plays a predominant role in the organization of the fibres.

In attempting to analyse the electrostatic contribution to the stability of the more complicated biogels, the investigator must consider the

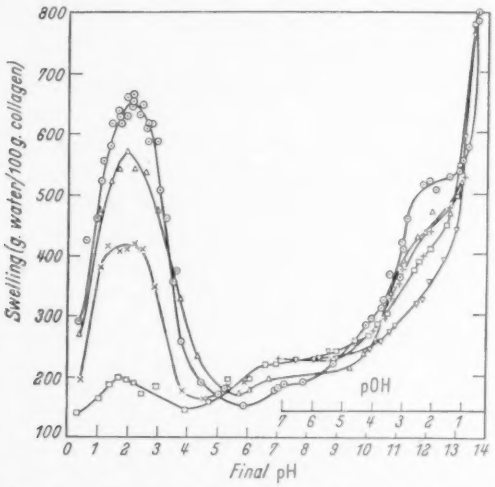

Fig. 5. Swelling of collagen D in hydrochloric acid (sodium chloride and sodium hydroxide) sodium chloride systems at 20°C: no salt; 0·05 M NaCl; 0·1 M NaCl; 0·2 M NaCl; 0·5 M NaCl; 2 M NaCl (from BOWES, J. H. and KENTEN, R. H.; *Biochem. J.* 46 (1950) 3)

influence of three variables: the degree of ionization of the different groups, which may be regulated by the exterior *p*H; the dielectric constant of the imbibing medium, which may be varied by organic solvents; and the salt concentration of the exterior solution.

Influence of *p*H—It is well known that the swelling of gelatin gels increases when the *p*H is shifted from the isoelectric point either to the alkaline or to the acid side. An analogous swelling behaviour of collagen at different *p*H can be seen in Fig. 5.

This increase in swelling capacity may be attributed to two factors (their quantitative evaluation will be discussed in the next chapter). The first factor is the accumulation in the gel of counter ions of the alkali or acid which penetrate the network and neutralize the fixed charges on the protein molecules. This accumulation of small ions in the gel increases its osmotic pressure and makes it swell by absorption of

water. The other factor, which is more pertinent to our discussion, is the change of the electrostatic potential of the macromolecules. Shifting the *p*H from the isoelectric point diminishes the number of attracting intermolecular "zwitterion" pairs and causes the attractive potential to be slowly replaced by one of repulsion. The number of electrovalent bonds therefore decreases and the network is loosened, permitting higher degrees of swelling.

We may assume that the first factor is the main contributor to the swelling of gelatin while both factors are operative in the swelling of collagen. The relative importance of the second factor may be deduced from the melting temperatures of the two substances. According to THEIS[68] the melting point of collagen is markedly dependent on *p*H and decreases on both sides of the isoelectric point with decrease in the number of electrovalent bonds.* On the other hand, the melting point of gelatin is remarkably constant in a wide range of *p*H and begins to sink only at extreme *p*H values, at which strong electrostatic repulsion forces become operative. The importance of *p*H in the primary formation of necklace fibrils from native serum proteins has been investigated by BARBU and JOLY[52] by means of streaming birefringence. As expected, they found that removing the *p*H from the isoelectric point diminishes the length of the fibrils because of the electrostatic repulsion acting against the van der Waals attraction.

New and interesting phenomena appear when the *p*H of the solution is sufficiently far removed from the isoelectric point to make the protein molecules behave like simple highly-charged polyelectrolytes. BARBU and JOLY[52] observed that at *p*H higher than 11·5 highly concentrated serum protein solutions yield gels without passing through the stage of necklace fibrillation. From measurements on double refraction of flow it was deduced that the intermolecular repulsion made the molecules swell, the gel being composed of an isotropic system of these highly swollen macromolecules. Similar phenomena were observed by the colloid chemists some twenty years ago in thixotropic gels of various proteins.[69] The theory advanced then to account for the thixotropy and structure of these gels was that the molecules are surrounded by thick hulls of solvation which interpenetrate and produce a hydration network. But as the thickness of the hydration shells had to be assumed to be a few hundred Å this theory seems to be untenable. KIRKWOOD[70] has shown that when a solute possesses a sufficiently high repulsive electrostatic potential there exists a critical concentration above which the solution ceases to be random and a periodic arrangement appears. This periodic arrangement, stabilized by occasional van der Waals bonding, is apparently the formative factor in concentrated, charged protein

* The decrease is still more pronounced at extreme values of *p*H, at which hydrogen bonds also are attacked by strong acidity or alkalinity.

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gels. The electrostatic-repulsion potential may be increased by a change in the second variable—*the dielectric constant*. Depressing the dielectric constant of the aqueous medium by addition of an organic solvent increases the electrostatic potential and strengthens the long-range orienting forces. KIRKWOOD and MAZUR[71] were able to obtain reversible gels from 40 per cent serum albumin at *p*H 7·5 by adding an equal volume of methanol. A similar effect of ethanol was observed with other proteins. MAGIS[51] found that the addition of ethanol to aqueous solutions of casein at *p*H 3–4 and at *p*H 9–12 reduced by two thirds the protein concentration required for gel formation.

Other gels in which the periodic arrangement is due to the high repulsive electrical potential, are the labile structures of the tactoid type. In those cases long-range forces, due to double-layer repulsion, orient and fix charged polyelectrolyte molecules.[72] Microscopic and ultramicroscopic examination of such systems reveals spindle-shaped regions of oriented disks or rods which may coalesce to form a separate anisotropic phase of high concentration. Striking phenomena of this type have been described for some inorganic sols where long-range forces act at a distance of 8000 Å.[73] Other investigations of gel-like structures due to double-layer interaction are those of tobacco mosaic virus (of a diameter of 150 Å and a length of 1500 Å) which arranges itself spontaneously in a hexagonal parallel packing.[74] This group of phenomena illustrates a transitory stage between sols and gels of polyelectrolytes and will not be followed up further.

Effect of salts—We shall consider now the third variable, the salt concentration. The influence of neutral salts on polyelectrolyte gels is of various kinds. Their concentration may play a decisive role in the transformation of polyelectrolyte sols into gels; also the Donnan distribution of salt between the existent gel and the exterior solution will be a prominent factor in determining the physical properties of the gels. These effects, apart from their purely electrostatic character, determined simply by the charge of the ions, are also specific, as expressed in the Hoffmeister series. The most important general effect of neutral salt is the formation of Debye atmospheres around the charged groups of the polymeric chains with a subsequent diminution of the electrostatic potential. Hence in the case of attraction at the isoelectric point the addition of salt decreases this attraction and diminishes the number of network junctions; correspondingly the swelling at the isoelectric point increases. On the other hand, in the *p*H region of electrostatic repulsion the screening effect of the salt weakens the repulsion, a higher number of cross-links, say of the van der Waals type, is made possible, and consequently a decrease in swelling takes place. At sufficiently high salt concentrations, Donnan effects (to be discussed in the next chapter) become negligible and need not be

taken into account. These considerations explain the swelling experiments on collagen in monovalent salts, represented in Fig. 5. The increase in swelling of the isoelectric collagen in the presence of salt proves that there is an orderly arrangement of charged groups stabilized by forces of attraction between them. Similar effects of salts on the balance between electrostatic repulsion and van der Waals bonding have been observed by the French investigators in their work on the fibrillation and network formation of globular proteins. By the combined action of salts and alcohols irreversible denaturation of the protein may occur with a corresponding opening up of the molecules and fibrillation. The newly-formed stretched molecular fibrils may be made to combine in a gel network if the attraction and repulsion are properly controlled by adjustment of *p*H, salt concentration, and dielectric constant. There will be a narrow range of gelation, and a range of over-attraction, when flocculation will result. An important study of denatured egg-albumin gels, in mixtures of water, *n*-propyl alcohol, and salt, is due to JIRGENSONS.[75] Gelation took place in 2 per cent solutions containing about 50 per cent alcohol at 0·2–0·5 molar sodium-chloride concentrations. The gels were thixotropic and melted at 67°C. Following JIRGENSONS' work, MEYERS and FRANCE[76] found that gels of denatured egg albumin may be obtained in salt solutions in 30 per cent acetic acid. These solutions differ from JIRGENSONS' in the fact that the acetic acid endows the fibrils with a high positive charge; they therefore resemble those obtained in very alkaline solutions. We have here a typical case of organization of the solutions by high electrostatic potential which is moderated by salt and permits van der Waals association.

The mechanism of gel formation from denatured proteins apparently always follows the pathway: native corpuscular protein → long denatured protein molecules → associated network. It seems that the interaction of detergents with proteins, which transforms the globular molecules into fibrils capable of gel formation and of extrusion as textile fibres, follows the same mechanism. It is assumed that the detergent salt penetrates into the protein molecules and ruptures the intramolecular salt bridges.[77]

Polyvalent salts—The action of polyvalent salt is more complicated than that of the monovalent. Highly ionized polyelectrolyte molecules exert sufficiently strong attraction on polyvalent ions of opposite charge to cause association[78] between the small ions and the polymeric chain; thus polyvalent ions influence the number of cross-links not only by changing the potential of the polyelectrolyte but by participating directly in the network junctions.

Gelation of polyuronic acids by polyvalent ions is of great practical importance. Thus both alginate fibres and pectin gels and jellies are cross-linked by calcium ions. WASSERMAN[79] has shown that the calcium

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ion associated with polyuronic acids may be exchanged with an excess of monovalent salt and from the exchange equilibria the strength of the bond estimated. Other industries making extensive use of cross-linking by polyvalent ions are the protein industries, of which the best example is the tanning of leather by means of basic chromates.

KLOTZ,[80] studying the binding of polyvalent and monovalent ions to proteins, has determined the bonding energy and its dependence on electrostatic and exchange contributions. He found that even in cases of strong bonding the major factor in the ion association is electrostatic attraction, which can be accounted for by the assumption that the added ions form dense Debye atmospheres around the charged groups of the protein.

The importance of the electrostatic cross-linking by polyvalent ions in biological structures can hardly be overestimated. In a study of the cell covers of amphibian eggs, HOLTFRETER[81] demonstrated that the rigidity of the cover is determined by the calcium content. Removal of the calcium by citrate causes a dissolution of the cover and the separation of the embryonic cells. The gel structure of the amoebian cortex is dependent on the cross-linking action of calcium ions, and its removal produces liquefaction of the plasmagel and inhibits the protozoan motility.[82] HEILBRUN[82] remarks that the calcium released as a result of fertilization or of artificial activation of the egg initiates the clotting or gelation which is responsible for the mitotic spindle. Finally, the gelation which follows stimulation is always accompanied by release of calcium ions. Apart from the biological aspects of these interesting observations, it is clear that their interpretation is closely dependent on the physico-chemical properties of the cross-linking of polyelectrolyte gels.

III. Equilibrium Properties of Polyelectrolyte Gels

The physical phenomena discussed in this chapter are connected with the solvent-gel interaction. From a fundamental approach to these interactions it is possible to deduce many of the characteristic properties of the gels, e.g. mechanical properties, their swelling pressure and stress-strain relations, the ion exchange capacity in salt solution, potentiometric titrations, and membrane potentials. We shall first deal with the interactions of gels with pure solvent, and as the most important solvent for polyelectrolyte gels is water, the larger part will be devoted to the study of aqueous solutions.

1. *Sorption*

The simplest method for the estimation of the gel-solvent interaction is to let the gel come to equilibrium with the solvent vapour, of pressure p, and to measure the regain, a, the amount of solvent absorbed per unit weight of dry gel. If the vapour pressure p of the gel is plotted against a,

from zero pressure to the vapour pressure p_0 of the pure solvent at a given temperature, a full sorption isotherm is obtained. This isotherm represents the vapour pressure of the gel as a function of gel concentration and is important for the evaluation of the thermodynamic properties of gels. The sorption isotherms of polymer and polyelectrolyte gels are smooth curves and it is difficult to distinguish any characteristic transition points between sorption proper and swelling or solution. In these gels no definite internal surfaces could be assigned on which absorption of solvent vapour might take place. The distinction, in our discussion, between sorption and swelling, is therefore made primarily for the sake of convenience and has no basic significance.

Although the sorption isotherms are continuous, their extremes—those of very low and of very high vapour pressures—show different and characteristic traits. The sorption of the first amounts of water is accompanied by strong liberation of heat, indicating the formation of true chemical compounds.[83] While the dielectric constant of highly-swollen gels approximates that of liquid water (80)—the dielectric constant of, e.g. slightly hydrated collagen is very close to that of ice—2. The strong binding of the sorption or hydration water may be revealed by direct mechanical tests. COMPTON[84] found that even pressures higher than 700 atmospheres could not press out the last 44–47 grams of water (per 100 g of dry fibre) absorbed on dry collagen. Following DOLE,[85] we therefore regard the sorption region as that part of the sorption isotherm in which the network arrangement is practically fixed and its entropy change is zero. The imbibed solvent in this region is immobilized on a fixed number of sites and its entropy contribution is correspondingly negative. With progressing sorption the sites become saturated and the network components are drawn apart; in the now mobile solvent the molecular chains mix and contribute positive entropy of mixing. This is the region of swelling.

In a recent investigation AUERBACH[86] demonstrated the existence of a transition point between two regions—say the sorption region and the swelling region—by measurements of rigidity modulus and dielectric constant of gelatin gels. Between 0 and 30 per cent concentration the elasticity follows the quadratic dependence on concentration and the dielectric constant is high, showing that this region is that of true swelling. At 30 per cent an abrupt increase in the rigidity occurs and simultaneously the dielectric constant drops rapidly, indicating that the water is immobilized in a manner characteristic of the region of sorption (Fig. 6).

Theoretical treatment—Sorption isotherms of polymer and polyelectrolyte gels are normally "S" shaped curves, some of which are represented in Fig. 7. A critical summary of newer work in this field will be found in the review of MCLAREN and ROWEN.[87]

The simplest theoretical approach to the analysis of the isotherms is that which assumes that LANGMUIR's absorption equation may be applied to gels. LANGMUIR[88] derived his formula for solid surfaces with a fixed number of sorbing sites without making any specific assumptions as to the surface arrangement of the sites. It was therefore natural to extend this treatment to a three-dimensional fixed array of sorbing sites to which free access of the sorbed molecules is permitted. J. J. HERMANS[89] and a group of Russian investigators[90] derived theoretically the three-dimensional equivalent of LANGMUIR's equation and

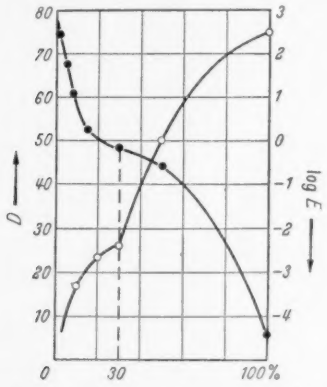

Fig. 6. The dependence of the modulus of elasticity (E) and the dielectric constant (D) of gelatin gels on concentration. The transition point between sorption and swelling is at concentration of 30 per cent (from AUERBACH, R. *Kolloid Z.* 124 (1951) 44)

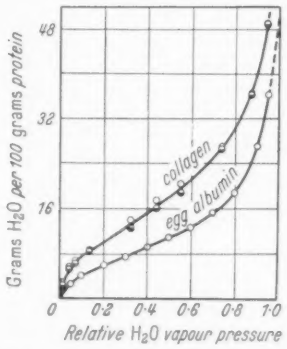

Fig. 7. Water-absorption curves for wet (open circles) and dry (half circles) collagen, and for lyophilized egg albumin at 25°C (from BULL, H.; *J. Amer. Chem. Soc.* 66 (1944) 1502)

applied it to the first part of the sorption isotherm. As might be expected the theory explains the experimental data satisfactorily in the range of monomolecular sorption, but for higher degrees of sorption another approach is required.

Better agreement with many sorption isotherms has been achieved by the application of the three-dimensional equivalent of the BRUNNAUER, EMMETT and TELLER[91] equation for multimolecular absorption. The extension of the multimolecular sorption isotherm to gels was carried out by CASSIE[92] and HILL,[93] using the methods of statistical thermodynamics. A more general treatment which includes both LANGMUIR and BET equations as special cases, was presented by DOLE.[94] BET's equation may be written in the following form

$$\frac{p/p_0}{n(1-p/p_0)} = \frac{1}{n_1 C} + \frac{C-1}{n_1 C} \cdot \frac{p}{p_0} \qquad \ldots.(4)$$

where n is the number of moles sorped and n_1 is the number of moles of sorption sites, C is a constant related to the energy of sorption. The plot of $1/n \cdot \frac{p/p_0}{1 - p/p_0}$ versus p/p_0 gives straight lines for a large number of polymers and proteins. From the slopes and the intercepts of the straight lines it is possible to evaluate C and n_1. BULL[95] found in his investigations on the sorption of proteins, that n_1, which should represent the area of the first sorption layer, is always much smaller than the continuous area of the protein as deduced from measurements of spread monolayers. PAULING[96] observed that if it is assumed that in the first stage of sorption each polar group of the protein binds a single molecule of water, good agreement between the number of sorption sites n_1 and the number of polar groups is obtained. In PAULING'S calculation it was assumed that the sorption takes place mainly on the amino, carboxylic, and hydroxylic groups and presumably on the carbonyl groups of proline and hydroxy-proline, while practically no absorption takes place on the peptide bond. The degree of agreement between n_1 and the number of polar groups may be deduced from Table I.

TABLE I

Protein	*Water adsorbed in the first layer moles*/10^5 g	*Number of polar groups moles*/10^5 g*
Ovalbumin crystallized . .	329, 342, 344	277–313
Gelatin, collagen	485, 529	328–609
Salmin	592	611–707
Serum albumin	374	424–424
β-Lactoglobulin crystallized . .	370	472–508

* Of each pair of numbers, the first number represents the polar residues not including the carbonyl groups of proline and hydroxy proline, and the second includes these.

In a more rigorous treatment each type of sorption should have been characterized by different BET constants but an attempt to generalize BET's equation in this direction did not lead to better agreement with experiment.

The successful application of the multimolecular sorption isotherm is limited to relative vapour pressures lower than 0·5–0·7. At higher vapour pressures solution or swelling in the proper sense cannot be neglected. WHITE and EYRING[97] extended HILL'S treatment to include swelling contribution but had to introduce empirical constants without definite physical meaning. The upper part of the sorption curve may be treated by direct application of the theory of concentrated polymer solutions.[98] The interesting theory of ROWEN and SIMHA[99] is a typical attempt in this direction and will be discussed below with the modern theories of swelling.

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A promising approach to polar sorption has been made by HOOVER and MELLON,[100] who consider explicitly the interaction of the polar groups of the proteins with the dipoles of water. According to the concepts of DE BOER and ZWIKKER[101] and of BRADLEY[102] the orientation of the first sorption layer extends far into the additional layers of water and imposes an ordered structure on the solvent in the gel. BRADLEY relates the relative vapour pressure and the regain, a, by the expression

$$\ln \frac{p_0}{p} = k_2 k_1^a \qquad \ldots.(5)$$

where k_2 and k_1 are temperature-dependent constants. HOOVER and MELLON were able to describe sorption isotherms of proteins and polymers for relative vapour pressures ranging between 4 per cent and 95 per cent. Mention should be made also of the sorption equation of HAILWOOD and HORROBIN,[103] who consider explicitly the interaction between the water and the sorbent. Although the agreement with experiment obtained by these authors is excellent, the use of a large number of equilibrium constants makes their theory unconvincing.

Thermodynamic considerations—After reviewing the work on the sorption isotherms we pass to the deduction of the pertinent thermodynamic functions from the isotherms and additional thermal measurements. KATZ,[104] in his classical researches on swelling, calculated the partial molal free energy and enthalpy of the solvent in the first stages of sorption and from his experiments concluded that the two magnitudes were equal. He therefore inferred that the entropy change accompanying initial swelling is practically zero and the whole inner energy of sorption may be transformed into available mechanical work. This conclusion was challenged by FRICKE and LÜCKE.[105] They proved by careful measurements that in general the enthalpy (ΔH) of sorption differs markedly from the free energy (ΔF), initial sorption being always accompanied by a strong decrease in the entropy of the solvent. The typical run of ΔH and ΔF versus amount of water in collagen is given in Fig. 8.

It will be observed that there may exist a certain point for which ΔH is equal to ΔF and ΔS is zero, but this occurs at higher degrees of sorption when most of the sorption heat has already been liberated. Beyond this point ΔS becomes positive, indicating that free mixing between the network chains and solvent occurs. Modern investigations of the thermodynamics of swelling of polar polymers, notably the work of MATHIEU[106] in France and SCHULTZ[107] in Germany on the swelling of nitrocellulose in acetone, and the analysis of DAVIS and MCLAREN[108] on the water sorption on proteins and polar polymers, confirm in principle the approach of FRICKE and LÜCKE. In all cases there is a

marked exothermal effect during the formation of the first sorption layer, in which definite compound formation between solvent and gel takes place. On further dilution of the gel the absolute value of ΔH decreases, the sorption now being regulated by the positive entropy of mixing.

2. *Swelling in pure solvent*

General Considerations—From the thermodynamic point of view the sorption isotherm already contains all the information about swelling

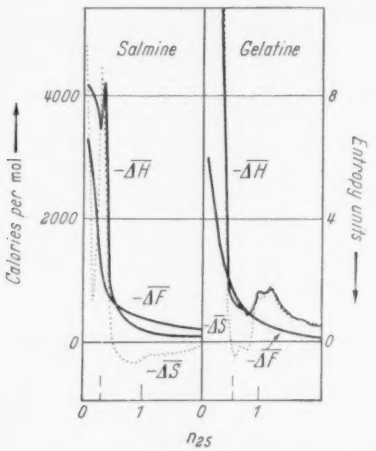

Fig. 8. Differential net heats ($-\Delta\bar{H}$), net entropies ($-\Delta\bar{S}$) and free energies ($-\Delta\bar{F}$) of sorption of water vapour on salmine and gelatin at 25°C. n-number of moles of water sorbed per 100 g gel. The vertical dotted lines on the n-axis represent the first layer of sorbed water (from DAVIS, S. and MACLAREN, A. D.; *J. Polymer Sci.* 3 (1948) 16)

pressures, and, as was shown by KATZ,[104] the relation between swelling pressure, P, and relative vapour pressure, $\frac{p}{p_0}$, is given by the expression

$$P = \frac{RT}{\bar{V}_s} \ln \frac{p_0}{p} \qquad \ldots.(6)$$

where $\bar{V}_s$ is the partial molal volume of the solvent. Yet it was tempting to measure the swelling pressures directly and in 1912 POSNJAK[109] carried out a series of measurements of swelling pressures in an apparatus similar to that used in osmometry. POSNJAK found that the relation between swelling pressure and gel concentration, c, obeys in many cases the simple empirical formula

$$P = P_0 c^k \qquad \ldots.(7)$$

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where P_0 and k are constants. For the gels investigated k lies between 2·5 and 4 while P_0 varies markedly with the nature of the substance. POSNJAK's equation was used extensively for the description of older swelling data. No important attempt had been made, however, to justify it theoretically until the advent of modern theories of polymer solubility and rubberlike elasticity.

FRENKEL[110] was the first to apply the new concept to lightly vulcanized rubberlike gels. His approach to a gel kept in equilibrium with pure solvent by an external pressure P (the swelling pressure) was based on two assumptions:

1. At high degrees of swelling the molecular chains, in spite of their cross-linking, mix freely with the solvent and contribute a positive mixing entropy. Denoting the free energy of mixing by F_{mix} we may attribute to the chains composing the network an "osmotic pressure"

$$-\pi = \frac{\partial \Delta F_{\text{mix}}}{\partial V} \qquad \ldots.(8)$$

where V is the swelling volume.

2. The swelling process distends the molecular network and stretches the macromolecules so that the entropy of the network decreases and its rubberlike resistance to stretch comes into play. If the stretching free energy is denoted by F_{stretch} the contractile pressure is

$$\mathscr{P} = \frac{\partial \Delta F_{\text{stretch}}}{\partial V} \qquad \ldots.(9)$$

The swelling pressure is related to the free energy difference ΔF of gel and solvent by

$$\frac{\partial \Delta F}{\partial V} = -P \qquad \ldots.(10)$$

Hence FRENKEL's assumptions are equivalent to

$$\frac{\partial \Delta F}{\partial V} = \frac{\partial \Delta F_{\text{mix}}}{\partial V} + \frac{\partial \Delta F_{\text{stretch}}}{\partial V} \qquad \ldots.(11)$$

or

$$P = \pi - \mathscr{P} \qquad \ldots.(12)$$

This formula expresses the idea that the swelling pressure is to withstand the osmotic pressure, which tends to make the gel imbibe the solvent; swelling pressure is aided by the elastic contractility of the network, which tries to press liquid out. At "equilibrium swelling"

$$P = 0, \text{ and } \pi = \mathscr{P} \qquad \ldots.(13)$$

It should be mentioned that in older papers we already find the osmotic pressure regarded as the driving force of swelling. Yet as the polymer

osmotic pressure was not sufficiently known and the elastic contribution not appreciated, these attempts did not lead to any conclusive results.

Swelling theory of non-polar gels—FLORY and REHNER,[40] J. J. HERMANS,[111] W. KUHN[112] and others[113] introduced the theoretical expressions for the free energy of mixing and stretch, derived from the statistics of polymeric chain molecules, into equation (12) and thereby obtained an explicit expression ready for comparison with experiment. The agreement of the measured swelling with that theoretically predicted[113] is, in general, good and lends strong support to the theory.

The osmotic pressure, π, for dilute gels, used by the above-mentioned authors, is

$$\pi = n \frac{RT}{V} \left\{ \frac{1}{Z} + \left(\frac{1}{2} - \mu' \right) \frac{1}{V} \right\} \qquad \ldots . (14)$$

where n denotes the number of monomeric units of network chains in the swollen volume V^*, Z is the degree of polymerization of each chain, and μ' is the Huggins interaction constant of solvent with polymer.

Before we introduce the equation for the elastic contribution, it is important to get a clear picure of the ideas underlying the discussion of the network contractility. These ideas will be used in the analysis of the behaviour of polyelectrolyte gels as well.

The fundamental assumption is that the total elasticity of the network is obtainable by the summation of the contributions of the individual molecules. Each macromolecule is assumed to be a chain composed of Z repeating monomeric units of length b. The thermal rotation of the atoms around the chemical single bonds permits the molecule to pass through an infinite number of configurations of varying probabilities. W. KUHN[115] characterized the configurations by the distances between the end atoms of the molecular chains. This permitted him to deduce a probability function for the end-to-end distances and to evaluate the most probable end-to-end distance for a chain of given degree of polymerization and monomer length. This most probable end-to-end distance of the unperturbed molecule—h_0—is found to be proportional to the square root of the number of monomers, Z, and to the length of the monomer, b. It is assumed that the chains in the unswollen network are joined by their ends in such a way that h_0 is the mean distance between cross-links.

When the gel is swollen from a volume of unity to a volume V and each dimension increases by a factor of $V^{\frac{1}{3}}$, the dimensions of each macromolecule increase too, according to the assumptions of the theory,

* As the volume of the dry gel was assumed to be 1 ml the equilibrium volume V denotes either the actual volume (in ml) or the degree of swelling (pure number) as the case may be.

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by the same factor. The distance between the cross-links in a swollen gel is now h and is equal to $h_0 \cdot V^{\frac{1}{3}}$. Stretching the end-to-end distance from h_0 to h brings the molecule into a less probable state by diminishing the number of available configurations (Fig. 9). Hence the decrease of

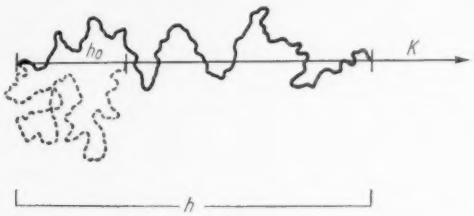

Fig. 9. Schematic representation of an unperturbed randomly-kinked molecule of end-to-end distance (h_0) and of a molecule stretched to end-to-end distance (h)

the network entropy mentioned above. The full calculation of the process leads to the expression for the elastic pressure contribution

$$\mathscr{P} = \frac{nRT^*}{ZV^{1/3}} \qquad \ldots.(15)$$

By introducing (14) and (15) into equation (12) we may obtain an expression for the swelling pressure. A more rigorous treatment[112], [40b] of the problem shows, however, that an additional entropy factor, the entropy of cross-linking, has to be taken into consideration. Summing up all the terms, we get for the swelling pressure

$$P = \frac{nRT}{ZV}\left\{\frac{1}{2} + \left(\frac{1}{2} - \mu'\right)\frac{Z}{V} - \lambda V^{2/3}\right\} \qquad \ldots.(16)$$

and correspondingly for equilibrium swelling when $P = 0$

$$Z = \frac{\lambda V^{5/3} - \frac{1}{2}V}{\frac{1}{2} - \mu'}$$

This is the equation we used on page 6 for the experimental determination of Z.

* Equation is valid only for low degrees of stretch. When the macromolecules are highly stretched a correction factor for the final length of the chain is to be included. This factor is called the inverse Langevin correction. The elastic pressure becomes

$$P = \frac{nRT\lambda}{ZV^{1/3}}$$

where λ is approximately

$$1 + \frac{0{\cdot}6\left(\frac{h}{Zb}\right)^2}{1 - \left(\frac{h}{Zb}\right)^2}$$

ROWEN and SIMHA[99] applied the same reasoning for the derivation of the sorption equation in the range of high vapour pressures when swelling becomes an important factor in the behaviour of the sorbent. Instead of the elastic contribution given above they use another expression suitable for lower degrees of swelling and containing an empirical elastic modulus. Although this approach seems to lead to satisfactory results in the case of non-polar polymers and isoelectric proteins, the justification for applying the equation of ROWEN and SIMHA to highly polar or ionized polymers is rather vague. It is permissible to neglect the polyelectrolyte behaviour in the region of low sorption where each absorbing group may be regarded as an independent unit. In dilute gels, however, the polymer electrostatic fields become so important that they should be included explicitly in the theoretical consideration. We pass now to the discussion of the polyelectrolyte effects and their introduction into the theory of gels.

Swelling of polyelectrolyte gels—The main difference between polyelectrolyte gels and the non-polar ones is the fact that the network of the ionized gel carries fixed charges. In the case of a non-isoelectric network in which there is a surplus of charges of one sign it is required that in the imbibing solvent be present an equivalent number of small free ions of opposite sign to maintain electro-neutrality. The presence of these "counter ions" and, in many cases, of other ions as well makes the osmotic balance in a polyelectrolyte gel entirely different from that in a non-polar gel in an organic solvent. Because of the extremely large number of small ions, as compared with the number of polymeric chains, their osmotic contribution will usually be so high that the osmotic pressure of the network discussed previously will be negligible.

About forty years ago PROCTER and WILSON[116] realized that the swelling of protein gels is determined mainly by the osmotic pressure difference between the ions in the gel and those outside. Yet the estimate of the osmotic contribution of the counter ions and other small ions does not follow from the classical laws of the ideal solutions. Even in the case of very dilute polyelectrolyte gels the deviations from ideality are very pronounced. KERN[117] found that the osmotic coefficient of sodium ions in the presence of ionized polyacrylic acid may drop to as low a value as 16 per cent of the ideal. The immobilization of the counter ions is exhibited also in other phenomena, for instance in the transference experiments of WALL.[118] The drop of the osmotic activity of the counter ions is due to the attraction of the powerful electrostatic field of the macromolecules. Hence in the theoretical consideration of the osmotic factor in the swelling of a polyelectrolyte gel we should include a correction which results from the deviation of the counter ion behaviour from ideality.

Another aspect of the polyelectrolyte nature of the gel is exhibited in

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the influence of the electrostatic field on the contractility of the network. The localization of a large number of ionized groups on a flexible randomly-kinked chain produces strong electrostatic repulsion which tends to stretch the macromolecular chains. This stretching effect has been amply demonstrated in viscometric and streaming birefringence measurements of polyelectrolyte solutions.[119] As the macromolecules of the gel are suspended in a medium containing counter ions, each fixed charge is surrounded by a Debye atmosphere which diminishes its repulsive action on the other fixed charges. The screening effect is dependent on the radius of the atmosphere; the closer the atmosphere the higher the screening and the smaller the repulsive energy. According to the classical theory of DEBYE and HÜCKEL the mean radius of the atmosphere is $\frac{1}{\kappa}$ where κ—a characteristic parameter of this theory—is directly proportional to the square root of the ionic strength. Hence with increasing concentration of the free ions $\frac{1}{\kappa}$ decreases, the atmosphere becomes denser and the repulsion diminishes. It is therefore seen that in dilute, highly ionized polyelectrolyte gels a strong stretching force develops along the chains and acts against the Brownian contractility of the network. On the other hand, in slightly swollen networks or in the presence of high salt concentrations the molecular chains exhibit their full elasticity as in the case of non-polar gels.

The qualitative picture drawn above may be replaced by a quantitative one if the electrostatic effects are derived from an electrostatic free energy which is added to the other free-energy contributions of the gel. During the last few years numerous formulae have been proposed for the free energy of polyelectrolytes, according to the molecular symmetry and range of concentration.[120] For our purpose the molecules are randomly coiled in a state of partial stretch, endowed with cylindrical symmetry, and suspended in solutions of quite high ionic strength. The equation of the electrostatic free energy of a single molecule for this case is

$$F_{e1} = \frac{\nu^2\varepsilon^2}{Dh} \ln\left(1 + \frac{6h}{\kappa h_0^2}\right)^* \qquad \ldots.(17)$$

where ν is the number of charged groups per macromolecule, ε is the charge of an electron, D the dielectric constant of the medium, and the other symbols as previously defined. The free electrostatic energy (F_{e1}) expresses all the electrostatic interactions between fixed and free ions per macromolecule; hence the total free electrostatic energy of the

* In the original paper of KATCHALSKY[119] h_0^2 was $\frac{2}{3}NA^2$ while here we define $h_0^2 = NA^2$. N is the number of statistical elements and A the hydrodynamic length of each element.

network is simply the product of F_{e1} by the number of molecular chains $\frac{n}{Z}$.

$$F_e = \frac{n}{Z}\frac{\nu^2\varepsilon^2}{Dh}\ln\left(1+\frac{6h}{\kappa h_0^2}\right) \qquad \ldots.(18)$$

and since in the swollen gel $h = h_0 V^{1/3}$

$$F_e = \frac{n}{Z}\frac{\nu^2\varepsilon^2}{Dh_0 V^{1/3}}\ln\left(1+\frac{6V^{1/3}}{\kappa h_0}\right) \qquad \ldots.(19)$$

The contribution of the free electrostatic energy to the swelling pressure should be included in (11) and (12) as an "electrostatic pressure" namely $\frac{\partial F_e}{\partial V}$. The "electrostatic pressure" may be resolved into two terms.

$$\frac{\partial F_e}{\partial V} = \left(\frac{\partial F_e}{\partial V}\right)_\kappa + \left(\frac{\partial F_e}{\partial \kappa}\right)_v \frac{\mathrm{d}\kappa}{\mathrm{d}V} \qquad \ldots.(20)$$

The physical significance of the terms on the right in this equation may be understood as follows. $\left(\frac{\partial F_e}{\partial V}\right)_\kappa$ is the change in the electrostatic energy with the stretch of the network at constant κ, i.e. at constant environmental conditions within the gel. It does not refer therefore to any osmotic factors operative in the medium, but it represents the electrostatic forces operative on the network itself. Hence this factor acts against the rubberlike contractility of the macromolecules. For reasons to be considered later we shall call this contribution the *mechano-chemical* factor of the gel.

The second term on the r.h.s. of equation (20), $\left(\frac{\partial F_e}{\partial \kappa}\right)_v \frac{\mathrm{d}\kappa}{\mathrm{d}V}$, measures the change of the electrostatic free energy with change in the ionic strength at constant network dimensions. This term is therefore the correction for the decrease in the osmotic pressure due to the attraction of the counter ions by the ionized polymolecules.

Now we are in a position to sum the terms and derive the swelling pressure of a polyelectrolyte gel:

$$P = \underbrace{\left\{\pi - \left(\frac{\partial F_e}{\partial \kappa}\right)_v \frac{\mathrm{d}\kappa}{\mathrm{d}V}\right\}}_{\substack{\text{ideal osmotic pressure} \\ \text{electrostatic depression} \\ \text{osmotic contribution}}} - \underbrace{\left\{\mathscr{P} + \left(\frac{\partial F_e}{\partial V}\right)_\kappa\right\}}_{\substack{\text{ideal elastic contractility} \\ \text{mechano-chemical stretch} \\ \text{elastic contribution}}} \qquad \ldots.(21)$$

Introducing the explicit equations for the osmotic pressure (comprising mainly the osmotic contribution of the small ions), for the elastic

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contractility, and for the free electrostatic energy, we obtain for gels swollen in pure solvent and ionized to a degree α:

$$P = \frac{n \,.\, RT}{V}\left\{\alpha\left(1 - B\sqrt{\frac{\alpha V}{n}}\right) - \frac{\lambda V^{\frac{2}{3}}}{Z}\right\} \qquad \ldots.(22)$$

Fig. 10–11. Pressure volume relation for swollen polymethacrylic acid gels at different degrees of ionization (α). Intercross-link degree of polymerization $Z = 370$

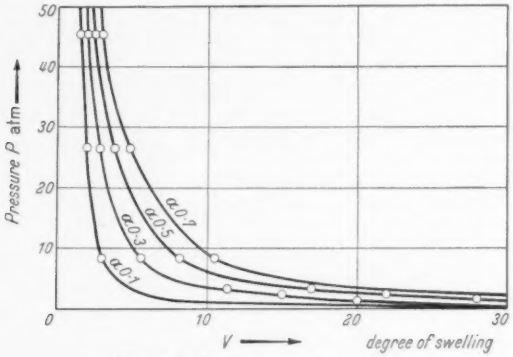

Fig. 10. Experimental results

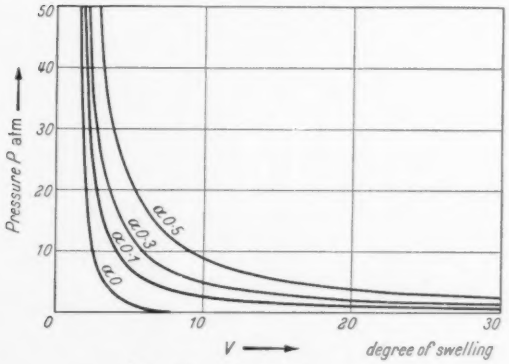

Fig. 11. Calculated curves

(from KATCHALSKY, A., LIFSON, S., EISENBERG, H.; *J. Polymer Sci.* 7 (1951) 571)

B is here a slowly-varying parameter, including the osmotic and contractile electrostatic contributions. The applicability of this theoretical treatment to experimental data was tested on slightly cross-linked gels of polymethacrylic acid.[114]

Polymethacrylic acid cross-linked by varying amounts of divinylbenzene was neutralized to different degrees by sodium hydroxide after thorough extraction of the unreacted material and drying. The cross-linked polymethacrylic acid was then swollen in pure water. Equilibrium swelling was attained after 3–4 weeks, as determined gravimetrically. Swelling pressures were measured directly in a cell of the Posnjak type and calculated from isopiestic determinations.

The results of the measurements given as swelling isotherms can be seen in Fig. 10. Fig. 11 gives the theoretical run for the gels investigated. The general agreement between the theoretical and experimental curves is good both in their general shape and in absolute values.

At high swelling pressures the ideal contractility term $\frac{\lambda V^{\frac{2}{3}}}{Z}$ becomes negligible and the swelling equation becomes

$$P = \frac{nRT\alpha}{V}\left(1 - B\sqrt{\frac{\alpha V}{n}}\right) \qquad \ldots\ldots(23)$$

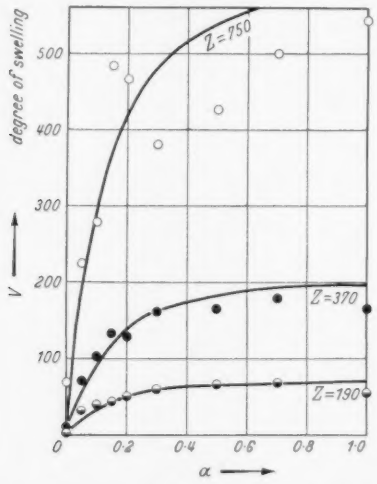

Fig. 12. Equilibrium swelling of gels of polymethacrylic acid cross-linked with divinyl benzene. Z degree of polymerization of intercross-linked chains; α degree of ionization of carboxylic groups; and V degree of swelling. Full circles—experimental results; curves—calculated from equation (24) (from KATCHALSKY, A., LIFSON, S., and EISENBERG, H.; *J. Polymer Sci.* 7 (1951) 571)

In very dilute gels the elastic contribution cannot be neglected and at equilibrium when $P = 0$ it becomes equal to the osmotic contribution.

$$\frac{\lambda V^{\frac{2}{3}}}{Z} = \alpha\left(1 - B\sqrt{\frac{\alpha V}{n}}\right) \qquad \ldots\ldots(24)$$

The agreement between theory and experimental data for equilibrium swelling can be seen in Fig. 12.

The experimental points are for three gels of different intercross-link chain lengths ($Z = 190$, 370, 750). The theoretical curves are calculated from equation (24). The spread of the experimental points at extremely high degrees of swelling is due to the difficulty of exact determinations in those cases in which the dry material in the gel constitutes only one half per cent ($V = 500$).

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Attention should be drawn to the fact that although the amount of network material in the gel is small, rigidity is maintained and the gels keep their shape even at high degrees of swelling. Thus the combined action of the powerful osmotic and mechano-chemical contributions of polyelectrolyte gels leads to an enormous swelling capacity, while the robustness of the polymeric chains guarantees mechanical rigidity.

3. *Behaviour in salt solutions*

Historically, the investigation of polyelectrolyte gels in salt solutions has contributed more than any other line of research to the understanding of gel behaviour. During the first quarter of this century Donnan advanced his famous equation for the distribution of ions over semipermeable membranes. Subsequently PROCTER[116] made use of this equation in his theory of swelling, which anticipates in many respects the modern view on gel behaviour. PROCTER assumed that the swelling of gelatin in salt solutions depends on two factors—the elastic contractility of the micellar structure and the osmotic pressure governed by the Donnan distribution of the ions. To make this plausible theory work, Proctor added two doubtful assumptions, neither of which has withstood recent criticism. One was that the elasticity of the network follows HOOK's law, regardless of the gel concentration, and the other, that it is possible to introduce into Donnan's equation ion concentrations instead of activities. PROCTER's theory was further developed by WILSON and WILSON[121] and it contributed to the understanding of the experimental data of LOEB[122] on protein gels.

In the discussion which arose around this theory many new and fruitful ideas were developed which are nowadays recast in modern form to account for the behaviour of ion exchangers. Thus GOSH[123] proposed to treat the salt accumulation in gels as an absorption phenomenon obeying LANGMUIR's isotherm; he proved that by this treatment a swelling equation equivalent to that of PROCTER and WILSON is obtained. The idea of GOSH has recently been taken up by BOYD[124] for the interpretation of ion exchange and further amplified by the statistical thermodynamic treatment of DAVIS.[125] Another approach to the interaction of salts with polyelectrolyte gels has been advanced by R. TOLMAN and A. E. STEARN.[126] Although severely criticized by their contemporaries, their paper contains many ideas which resemble the modern approach to polyelectrolytes and polyelectrolyte gels. In the remarkable summary of their paper these authors state, referring to protein gels:

"... (2) In an acid solution, the adsorbed hydrogen ions, together with a corresponding number of anions, form a "double layer" on the walls of the pockets or pores in the interior of the gel and this leads to swelling and imbibition of water by electrostatic repulsion.

(3) The addition of a strong electrolyte to such a swollen colloid, either a neutral salt or excess of strong acid which caused the original swelling, will furnish ions in the interior of the pockets which will tend to arrange themselves so as to neutralize the electrical fields of the adsorbed layer and thus bring about a reduction of swelling.

(4) The addition of a neutral salt to an acid solution tends to neutralize the electrical field . . .

(5) It must further be noticed that in accordance with our theory salts with polyvalent ions should be more effective in reducing swelling than salts with univalent ions . . . "

It is readily seen that TOLMAN failed to appreciate the great importance of Donnan distribution and did not succeed in giving his theory a mathematical formulation. Modern views combine the concepts of both PROCTER and TOLMAN and take notice of all three factors: the elastic contractility, corrected for concentration effects; the Donnan distribution, determinating the osmotic contribution; and the electrostatic field effects which determine the activity of the imbibed ions and the macromolecular repulsion.

A few years ago VERMAAS and J. J. HERMANS[127] made an important attempt to improve PROCTER's theory by applying the modern expressions for the osmotic pressure of the macromolecules and the contractility of the network. They used the approximate Donnan equation without introducing appropriate activity factors for the counter ions in the charged gel. Nevertheless, VERMAAS and HERMANS succeeded in describing very satisfactorily the equilibrium volumes of highly swollen xanthogenates in the presence of numerous monovalent and polyvalent neutral salts.

The major part of recent research on the interaction of gels and salt solutions is devoted to the study of ion exchange reactions. Although these studies have been carried out in highly cross-linked polyelectrolyte resins of small swelling capacity and under physico-chemical conditions far removed from ideality, they contain much valuable material for a general understanding of the behaviour of polyelectrolyte gels. When an acidic ion-exchange resin is brought into contact with a mixture of two salts, some of the cations and anions penetrate into the resinous gel phase. In order to keep electroneutrality, the number of entering cations must be balanced either by an equivalent number of anions entering the gel or by the expulsion, into the exterior solution, of an equivalent number of cations from the gel. The second possibility has been described by earlier workers[128] as a methatetic reaction between outer ions and inner ions:

$$A_i + B_0 \rightleftharpoons A_0 + B_i \qquad \ldots\ldots(25)$$

the electroneutrality providing for the stoichiometric relations. To this reaction an equilibrium constant was assigned, of the form

$$\frac{a^{\circ}_{A^+} \cdot a^{i}_{B^+}}{a^{i}_{A^+} \cdot a^{\circ}_{B^+}} = K \qquad \ldots\ldots(26)$$

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where the a's denote the activities of the corresponding ions participating in the exchange. If the bonding is assumed to be purely electrostatic and the specific differences between the ions are disregarded it is plausible to assume that the work of transport of a mol-ion of type A into the gel is equal and opposite in sign to the work of transferring a mol-ion of B out of the gel. Hence the change in standard free energy accompanying reaction (25) is zero and the equilibrium constant $K = 1$. Yet many authors went even further and substituted concentrations for activities in equation (26) and obtained:

$$\frac{c^{\circ}_{A^+} \cdot c^{i}_{B^+}}{c^{i}_{A^+} \cdot c^{\circ}_{B^+}} = D \qquad \ldots\ldots(27)$$

for which the so-called selectivity coefficient D is neither unity nor constant, although in many cases quite close to unity.

The rigorous thermodynamic treatment of DONNAN and GUGGENHEIM[129] for the distribution of ions between gel and exterior solution permits the deduction of exact equations which avoid unnecessary approximations. Their only restriction is that the ions in the gel phase do not undergo neutralization as in the case of hydrogen and hydroxyl ions discussed below. For the sake of simplicity we shall give the equation only for monovalent ions.

For the distribution of a single salt the equation of DONNAN and GUGGENHEIM gives the following relation between the activities of the ions (a), the osmotic pressure (P), and the partial volume of the solvent ($\bar{V}_s$).

$$\frac{P\bar{V}_s}{RT} = \ln \frac{a^{\circ}_{+} \cdot a^{\circ}_{-}}{a^{i}_{+} \cdot a^{i}_{-}} \qquad \ldots\ldots(28)$$

The corresponding relation for the ion exchange of cations as given by DONNAN[130] is

$$\frac{P(\bar{V}_{A^+} - \bar{V}_{B^+})}{RT} = \ln \frac{a^{\circ}_{A^+} \cdot a^{i}_{B^+}}{a^{i}_{A^+} \cdot a^{\circ}_{B^+}} \qquad \ldots\ldots(29)$$

where $\bar{V}_A$ and $\bar{V}_B$ are the partial molal volumes of the cations A and B respectively. Thus the specificity of the ions is expressed both in their activities and in their specific volumes. In general, the terms on the left-hand side of equations (28) and (29) are practically equal to zero, so that we obtain the usual expressions for Donnan equilibria

$$\frac{a^{\circ}_{+} \cdot a^{\circ}_{-}}{a^{i}_{+} \cdot a^{i}_{-}} = 1 \qquad \ldots\ldots(28')$$

and

$$\frac{a^{\circ}_{A^+} \cdot a^{i}_{B^+}}{a^{i}_{A^+} \cdot a^{\circ}_{B^+}} = 1 \qquad \ldots\ldots(29')$$

equation (29′) being the equivalent of the methatetic exchange reaction (26). On the other hand, if the pressure term in equation (29) is regarded not as the external pressure but as the contractile pressure of the gel, this term may not be neglected. Recently GREGOR[131] made use of this approach in the construction of a new thermodynamic theory of ion exchange.

The simplified equation (27) results from equation (29′) when activities are expressed in terms of concentration (c), and activity factors (f).

$$\frac{c^{\circ}_{A^+} \cdot c^{i}_{B^+}}{c^{i}_{A^+} \cdot c^{\circ}_{B^+}} = \frac{f^{i}_{A^+} \cdot f^{\circ}_{B^+}}{f^{\circ}_{A^+} \cdot f^{i}_{B^+}} = D \qquad \ldots . (30)$$

Hence the theoretical evaluation of the selectivity coefficient, (D), depends on the correct estimate of the activity factors. It is clear that as long as only pure electrostatic influences are considered, no great difference is to be expected among the activity factors of different monovalent ions; thus $f^{i}_{A^+} \sim f^{i}_{B^+}$, $f^{\circ}_{A^+} \sim f^{\circ}_{B^+}$ and $D \sim 1$. The selectivity coefficient (D), being dependent on the ratio of activity factors, is less sensitive to their absolute value than is the distribution of a single salt, determined by equation (28′). An inspection of the values of (D) may be misleading in this respect and may suggest that the activity factors in the gel are close to unity. However, direct measurements by KERN[132] of the activity factors of sodium ions in the presence of polyacrylic acid prove that their deviation from unity is very large. At higher degrees of ionization, such as occur with most ion exchangers, the factor drops to about the same value as the osmotic coefficient, i.e. to about 16 per cent of the ideal. Various attempts have been made to describe the activity coefficients by empirical or semi-empirical formulae which are useful in technological practice. As this review is not concerned with the practical application of polyelectrolyte gels we shall not consider these formulae. Rather we shall discuss here the case of more dilute gels, which are of less practical importance but for which the polyelectrolyte theory permits the evaluation of activity factors and Donnan distributions of ions between gel and solution. Typical cases to which the discussion applies are the slightly cross-linked polyacrylic and polymethacrylic acids, the polysterene sulphonic acids, and the highly swollen xanthates.

In accordance with the classical method of DEBYE, it is found that the activity factors of the counter ions are derived from the electrostatic free energy by the relation

$$RT \ln f_i = \left(\frac{\partial F_e}{\partial n_i}\right)_{n_i} \qquad \ldots . (31)$$

where n_i denotes the number of moles of counter ions. The validity of the theoretical equation for the description of the Donnan distribution

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of sodium chloride between ionized polymethacrylic acid solution and pure salt solution has been tested by KEDEM.[133] The experimental results given in Fig. 13 are in good agreement with those of KERN and demonstrate clearly the general run of the activity coefficients in polyelectrolyte solutions: the values of f_i *decrease* with increasing ionization of the polymer and with polymer concentration but *increase*

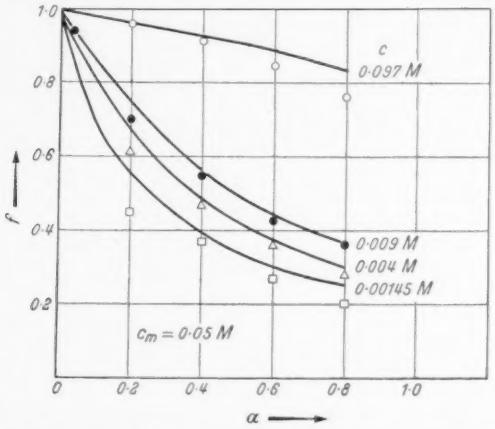

Fig. 13. Activity coefficient (f) of gegen ions (Na) in polymethacrylic acid solutions. The polymer concentration is the same throughout ($C_m = 0{\cdot}05$ M in monomoles). The variables are the degree of ionization (α) and the concentration of neutral salt (NaCl 0·001145 M, 0·004 M, 0·009 M and 0·097 M) (from KATCHALSKY, A. and KEDEM, O.; Unpublished results)

with increasing salt concentration, i.e. with progressive screening of the fixed charges.

From equation (31) the activity coefficients of the counter ions of the gel are found to be

$$kT \ln f_i = -\frac{\nu^2 \varepsilon^2}{D\hbar_0 \left(V^{1/3} + \frac{\kappa h_0}{6} \right)} \frac{c_m}{\Sigma c_i} \qquad \ldots\ldots(32)$$

where c_m denotes the concentration of network material of the gel and Σc_i the total concentration of free ions in the gel.

This equation has been tested by MICHAELI[134] for polymethacrylic-acid gels of intercross-link molecular weight $Z = 195$. Fig. 14 represents the equilibrium swelling volume (V) as a function of the degree of ionization of the gel and of the exterior salt concentration. In Fig. 15 are given the salt concentrations in the gel found experimentally as well as the corresponding theoretical curve calculated with the aid of equation (32).

Although the approximate activity factors given by the theory hold

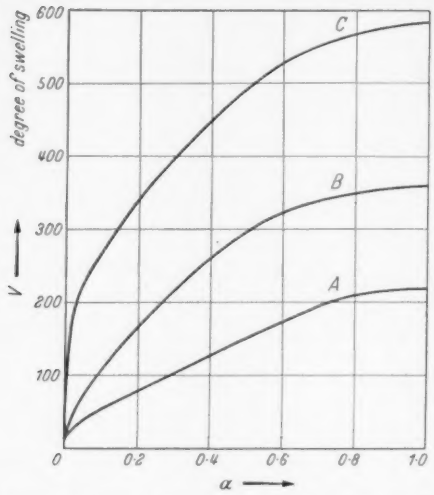

Fig. 14. Equilibrium swelling of ionized, cross-linked polymethacrylic acid in salt solutions. (*A*) external solution 0·55 M NaCl, (*B*) external solution 0·11 M NaCl, (*C*) external solution 0·007 M NaCl. The degree of polymerization of the intercross-link chains in these gels is *Z* 195. *V* is the degree of swelling, α the degree of ionization (from KATCHALSKY, A. and MICHAELI, I.; In preparation)

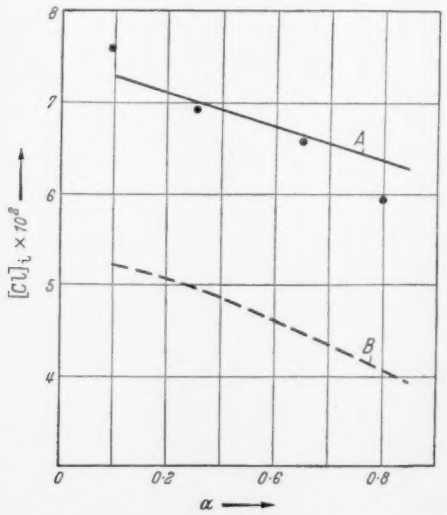

Fig. 15. Salt concentrations in polymethacrylic-acid gels of different degrees of ionization (α) in equilibrium with an external salt solution of 0·11 M NaCl. (*A*) full circles—experimental results; solid line—theoretical. (*B*) dotted line —calculated from DONNAN'S equation with activity factors assumed equal to unity (from KATCHALSKY, A. and MICHAELI, I.; In preparation)

for very dilute gels they fail to describe more concentrated systems where specific factors become operative. It may be that with the development of a polyelectrolyte theory that takes notice of the final radius of the ions a better approach for higher concentrations will be found. Unfortunately no such general theory exists at present and we must be content with a qualitative description of the data.

Since the classical research of HOFMEISTER and his school[135] on the effect of salts on colloidal systems, an extensive literature has accumulated which cannot be explored in this review. Only a few more general facts will be chosen for discussion.

It was found that as a rule salts which act as *salting-in* agents accumulate readily in the gel and increase its swelling, while those which act as *salting-out* agents accumulate to a lesser degree and often effect shrinkage of the gel. The relation between the tendency to accumulate and the influence on swelling has been elucidated thermodynamically by POLANYI.[136]

A typical series of experiments[137] representing the direct relation between absorbability of different salts and influence on swelling is given in Table 2.[138]

TABLE 2

Swelling of pretreated gelatin (grams of sorbed water/100 g dry gelatin) in different salt solutions. The bracketed figures represent the swelling while the series give the accumulation of salt

Potassium salts .	KCNS > (373)	KI > (257)	KBr > (78)	KNO > (145)	KCl > (69)	K_2SO_4 (41)
Lithium salts .	LiJ > (182)	LiBr > (123)	LiCl > (70)	Li_2SO_4 (41)		
Chlorides . .	$BaCl_2$ > (240)	$CaCl_2$ > (104)	LiCl > (70)	$MgCl_2$ > (85)	NaCl > (69)	KCl (69)
Sulphates . .	$MgSO_4$ > (38)	Li_2SO_4 > (41)	K_2SO_4 (41)			

The specific effect of the salts on swelling has its counterpart in the change of melting point and elastic modulus. As may be expected, strongly absorbed salts depress the melting point[139] and diminish the rigidity[140] of the gels while salts which exhibit poor imbibition either do not influence the melting point or even increase it. The ions most active in favouring swelling and in decreasing melting point and rigidity of collagen and gelatin are the CNS^- and I^- among the anions and Ca^{++}, Ba^{++}, Mg^{++}, and Li^+ among the cations.[141] The least absorbed ions, which may increase the melting point and rigidity, are SO_4^{--} and in some cases CO_3^{--} and $S_2O_3^{--}$.[142] Lyotropic series have been found also in ion-exchange phenomena[24] and a series of decreasing cation selectivity is given on p. 36.

$Th^{++++} > Hf^{++++} > Zr^{++++}$

$Ac^{+++} > La^{+++} > Y^{+++} > Sc^{+++} > Al^{+++}$

$Ba^{++} > Sr^{++} > Ca^{++} > Mg^{++} > Be^{++}$

$Ag^{+} > Tl^{+} > Cs^{+} > Rb^{+} > NH_4^{+} > K^{+} > Na^{+} > H^{+} > Li^{+}$

There is doubtless a relation between the degree of hydration of the ions and their lyotropic activity but this fact has not been elucidated in a satisfactory quantitative theory. In many cases a correlation has been found between the radius of the hydrated ions and their position

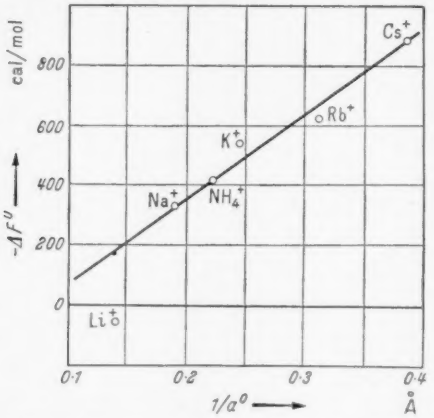

Fig. 16. Correlation of the standard free energy ($-\Delta F°$) of the exchange reaction: $A^+ + HR \rightleftharpoons AR + H^+$ in zeolite formation with the reciprocal radius of the hydrated cations $\frac{1}{a^\circ}$. $a°$ is deduced from the DEBYE-HÜCKEL theory and expressed in Ångstroms. $-\Delta F°$ is given in cal/mol (from BOYD, G. F., SHUBERT, J. and ADAMSON, A. W.; *J. Amer. Chem. Soc.* 69 (1947) 2828)

in the Hoffmeister series. Generally the swelling activity of the monovalent cations increases with decreasing ionic radius in solution. The behaviour of the anions is more complicated and in many cases the activity seems to decrease with the radius. Empirical relations for the dependence of salt imbibition on the ionic radius have been established by BUCHNER and his co-workers.[143] Similar observations have been made with acidic resin exchangers on the effectiveness of cation exchange. NACHOD and WOOD,[144] working with coal treated with sulphuric acid, found a linear decrease of the exchange capacity with increasing radius of the hydrated ions. An important contribution to the quantitative formulation of the lyotropic effects in ion exchange has been made by BOYD *et al.*[124] By a study of the change in the standard free energy accompanying the exchange equilibria, $A^+ + HX \rightleftharpoons H^+ + AX$, it was possible to define the absorption affinity of a series of cations to the acidic resin and to correlate the affinity with the charge and radius of

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the cations. BOYD found that in a series of monovalent cations the standard free energy increases more or less linearly with the reciprocal of the radius of the hydrated ion, as shown in Fig. 16.

Potentiometric titration—This survey has covered hitherto mainly the interaction of polyelectrolyte gels with salts that do not interact chemically with the gels to produce covalent bonds. The ions discussed in the previous paragraph form electrostatic bonds only, and in the case of polyvalent ions give rise to associated ion pairs. Yet two ions which produce covalent bonds—the hydrogen and hydroxyl ions—deserve special consideration. As polyelectrolyte gels carry fixed acidic

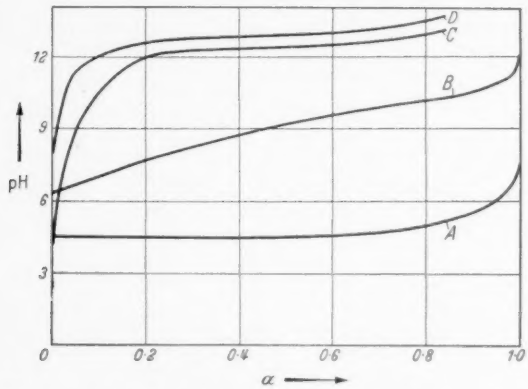

Fig. 17. Potentiometric titration curves of ion-exchanger gels with acidic groups of different strength. (*A*) Ionizable groups—SO_3H, (*B*) ionizable groups—COOH, (*C*) ionizable groups resorcinol—OH, (*D*) ionizable groups phenolic—OH, (from TOPP, N. E. and PEPPER, W.; *J. Chem. Soc.* (1949) 3300)

or basic groups the interaction with H^+ is of importance not only from the point of view of the physical phenomena accompanying ion imbibition, but mainly as a means of determining the number of functional groups in the network. The determination of acid-base binding capacity of biogels and ion-exchange resins is of the utmost importance for the specification of the gel properties.

In order to obtain a more detailed description of the gel acid-base binding capacity recourse must be taken to potentiometric titration, which gives information not only about the number of reactive groups but also about their dissociation constants.[145] A typical series of potentiometric titrations of gels of weak, medium, and strong acidic properties is assembled in Fig. 17.

Similar titration curves are obtained for polybase gels and for the polyampholyte protein gels. Of special interest is the characterization of acidic and basic groups in permselective membranes by potentiometric titrations which resemble those of other polyelectrolyte gels.[146]

The potentiometric titration curves of gels, which relate the *p*H of the exterior solution to the degree of ionization of the gel, resemble the titration curves of monofunctional acids or bases. However, the "dissociation constants" differ often by two orders of magnitude from the expected value for the functional group, and the slope of the curves is not the usual one. Addition of neutral salt[147] changes the picture markedly and brings the curves closer to the expected run. In the case of weak or medium polyacid gels the *p*H at half neutralization is always much higher than that of the monofunctional group, while in the presence

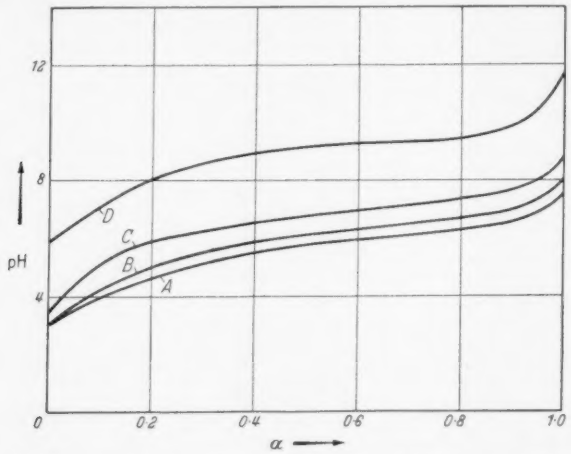

Fig. 18. Potentiometric titration of polymethacrylic-acid ion-exchangers in the presence of different salt concentrations. (*A*) salt concentration in exterior solution 2 M NaCl, (*B*) salt concentration in exterior solution 1 M NaCl, (*C*) salt concentration in exterior solution 0·1 M NaCl, (*D*) in absence of NaCl (from HALE, D. K. and REICHENBERG, D.; *Discuss. Faraday Soc.* No. 7 (1949) 82)

of salt the *p*H is depressed and comes closer to that characteristic of the group. This is shown clearly in Fig. 18.

The potentiometric behaviour of polybases is opposite to that of polyacid gels. While the *p*H at half neutralization (representing the apparent dissociation constant) is much lower than expected, it is increased by the addition of salt. Protein gels[148] show the combined effect of both acid and basic groups; in the acid range they behave as polybase gels and in the alkaline range as polyacid gels.

The importance of these titrations for the structural analysis of various biogels lends special interest to their fuller theoretical analysis. The potentiometric determination of functional groups is a standard procedure in the investigation of soluble polyelectrolytes, and this procedure is now slowly developing into a means of defining solid structures of gel-like character as well.

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As the description of the gel-titration in the absence of salt is slightly more complicated than in the presence of salt, we shall discuss only potentiometry in the presence of salt. Let us consider the alkaline titration of a weak polyacid gel in the presence of sodium chloride. At every point of the titration the distribution of the hydrogen ions is correlated with that of the chloride ions by the Donnan relation

$$\frac{a^{\circ}_{H^+}}{a^{i}_{H^+}} = \frac{a^{i}_{Cl^-}}{a^{\circ}_{Cl^-}} \qquad \ldots.(33)$$

which gives, when we take negative logarithms and substitute $p\mathrm{H}$ for $-\log a_{\mathrm{H}}$,

$$p\mathrm{H}^{\circ} = p\mathrm{H}^{i} + \log \frac{a^{\circ}_{Cl^-}}{a^{i}_{Cl^-}} \qquad \ldots.(34)$$

This equation permits the evaluation of the interior $p\mathrm{H}^{i}$ when the exterior $p\mathrm{H}^{\circ}$ and the distribution of salt between gel and solution are known. As has been pointed out above, the activity factor of the counter ions drops markedly in the presence of polyelectrolyte. The activity factors of ions of the same sign as the fixed charges, however, are slightly increased and are practically equal to unity. Hence

$$\frac{a^{\circ}_{Cl^-}}{a^{i}_{Cl^-}} = \frac{c^{\circ}_{Cl^-}}{c^{i}_{Cl^-}} \qquad \ldots.(35)$$

so that the concentration ratio may be used in place of the activity ratio in the evaluation of $p\mathrm{H}^{i}$.

The behaviour of hydrogen ions in the gel is very similar to that in polyelectrolyte solutions and the same theoretical treatment may be applied. According to modern theories[149] the $p\mathrm{H}$ of a polyelectrolyte solution is dependent on the intrinsic dissociation constant ($p\mathrm{K}_0$) of the functional group—which is normally that of a monomeric unit—on the degree of ionization (α) and on the potential (ψ) of the molecular surface. The potentiometric equation for polyacids is:

$$p\mathrm{H} = p\mathrm{K}_0 - \log \frac{1-\alpha}{\alpha} + \frac{0{\cdot}4343\varepsilon\psi}{kT} \qquad \ldots.(36)$$

and a corresponding equation for polybases is:

$$p\mathrm{H} = p\mathrm{K}_0 - \log \frac{\beta}{1-\beta} - \frac{0{\cdot}4343\varepsilon\psi}{kT} \qquad \ldots.(37)$$

where β is the degree of ionization of the polybase. The potential may be derived from the electrostatic energy (F_e) by the simple expression

$$\varepsilon\psi = \left(\frac{\partial F_e}{\partial \nu}\right)_{\kappa} \qquad \ldots.(38)$$

where ν, as before, is the number of ionized groups per macromolecule.

In order to justify the application of equations (36) and (37) to gels we must show that the electrostatic potential of the molecular chains of the network is practically the same as that of free macromolecules in solution. An indirect proof is found in the conductimetric measurements of TAFT and MALM[150] on gelatin. These workers found that because of the electrostatic attraction the conductivity of the free small

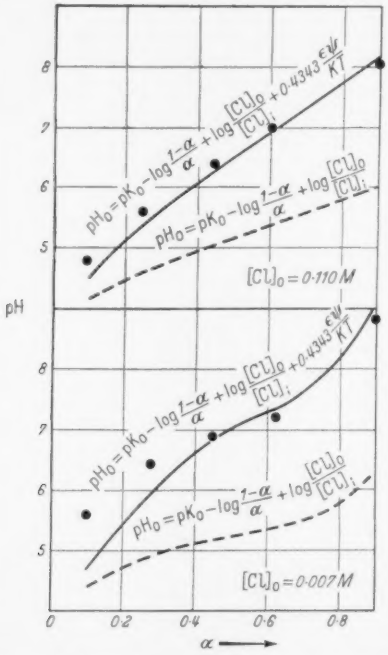

Fig. 19. Potentiometric titration of highly swollen polymethacrylic-acid gels ($Z = 195$) in the presence of 0·007 M NaCl and 0·110 M NaCl in exterior solution. Full circles indicate experimental results; solid curves are calculated from equation (39) with inclusion of the potential correction, $\varepsilon\psi$; dotted curves, calculated ideal curves for the corresponding gels and degrees of swelling with neglect of the potential term (from KATCHALSKY, A. and MICHAELI, I.; In preparation)

ions is less in the presence of gelatin than in free solution. But the sol-gel transition of the gelatin hardly affects the conductivity, indicating that the polyelectrolyte field acting on the ions is the same in the sol and gel phases.

Introducing into equation (34) the expression for $p\mathrm{H}^i$ from (36) and for the anion activities from (35), we get for the titration of a polyacid gel in the presence of salt:

$$p\mathrm{H}^\circ = p\mathrm{K}_0 - \log\frac{1-\alpha}{\alpha} + \log\frac{c^\circ_{\mathrm{Cl}^-}}{c^i_{\mathrm{Cl}^-}} + \frac{0{\cdot}4343}{kT}\left(\frac{\partial F_e}{\partial \nu}\right)_\kappa \qquad \ldots.(39)$$

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Simplified equations, neglecting the potential term $\left(\frac{\partial F_e}{\partial v}\right)_\kappa$ have been used in the analysis of gel titrations. It should be clear, however, that at low salt concentrations and in highly swollen gels this term may contribute up to two pH units and its neglect may lead to significant errors.

Equation (39) gives an explanation for the curves obtainable in different salt solutions (Fig. 18).

At low salt concentrations the potential is high and the Donnan distribution requires $c^\circ_{Cl} > c^i_{Cl}$. Hence the two last terms on the right-hand side of equation (39) make a positive contribution to the pH. With increasing salt concentration the potential goes to zero and c°_{Cl} approaches c^i_{Cl}, so that both terms diminish and the titration curve approaches that of a monofunctional acid. An inspection of equation (37) leads to the conclusion that in case of polybasic gels the pH should exhibit opposite behaviour.

The quantitative applicability of equation (39) was tested on the dilute polymethacrylic-acid gels described above.[134] Fig. 19 gives potentiometric titrations of the gels along with the calculated curves. The degree of ionization (α) was assumed to equal the ratio of the concentration of the adsorbed alkali to the polymer concentration, and $p\mathrm{K}_0$ was taken as 4·86, the value found from titration of polymethacrylic acid solutions.[149] It will be observed that the general agreement between theory and experiment is good; moreover, the importance of the potential term is clearly demonstrated.

IV. Mechano-chemistry of Polyelectrolyte Gels

After reviewing some of the principal aspects of the behaviour of polyelectrolyte gels we pass to one of the most intriguing phenomena of nature—the transformation of chemical into mechanical energy by some biogels and synthetic gels. This process underlies all motility of living organisms and is a fundamental distinction between the motion of living matter and that of technological devices. A cursory survey of living beings shows that none of the animals, plants, or protista moves according to the principles of thermal or electrical machines. The working principle of animate matter is always mechano-chemical, i.e. based on the direct mechanical utilization of chemical energy in isothermal or nearly isothermal cycles. The driving mechanisms of living matter are always biogels of pronounced polyelectrolyte nature, varying in type and organization. Different organisms utilize various gel mechanisms for their motility: the amoeboid movement is based on progressive sol-gel transformation, the movement of the plants is founded on reversible swelling and shrinkage of protoplasm, while the great majority of higher animals and many protista use an intricate

and very effective contractile gel-mechanism. Recent investigations of muscular contractility have made the contractile machine of higher animals amenable to physico-chemical research by reducing it to elementary molecular components. The principles underlying the behaviour of the contractile mechanisms of the animate beings are partially covered by the previous discussion of polyelectrolyte gels but many unknown phenomena require further elaboration to make the picture of the muscular work complete and consistent.

Already at the end of the nineteenth century many of the physiologists realized that in order to understand biological motility, it is necessary to consider the behaviour of gels.[151] Yet their attempts were directed at gels of low swelling, for which the adsorption of water is of low reversibility and which cannot provide the basis of highly efficient cycles of the muscle type, in which almost 50 per cent of the chemical energy is transformed into work.[152] After a long interruption, during which major strides were made in the elucidation of the biochemical metabolic changes in the muscle[153] and its quantitative energetics were worked out,[154] a dramatic return to the colloid chemistry of the muscle has taken place. The isolation of various muscle proteins and the realization that myosin is the main structural component led to its physico-chemical characterization by VON MURALT and EDSALL.[155] Subsequently, H. H. WEBER[156] produced "synthetic" myosin fibres which exhibited some of the properties of muscle fibrils. This line of research culminated in SZENT-GYORGYI'S discovery that the main components of the muscular structure are two proteins, the actin and the myosin (L-myosin), which may be combined in the form of actomyosin. Addition of adenosine-triphosphate to threads of actomyosin gels results in a rapid contraction resembling that of the native muscle.

Remarkable is the universality of the contractile constituents in the animal kingdom and the widespread utilization of the mechanism. Actin and myosin from quite different animals combine to give actomyosins and all known actomyosins behave similarly towards ATP.[157]

The details of this important work are summarized in several books and reviews by SZENT-GYORGYI himself and by his followers[8] and a recent extensive review by H. H. WEBER[158] summarizes critically all pertinent information to date. We choose here only a few observations required for the following discussion of synthetic mechano-chemical gels.

The gelation properties and the contractility of actomyosin are best discussed in terms of its constituents. The myosin component is a long-chain molecule of molecular weight between 840·000 and 858·000 with a long axis of 1500–2300 Å and thickness of about 22–24 Å. It contains a remarkably high percentage of dibasic and diacidic amino-acid residues, which endow it with a pronounced polyelectrolyte character.

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Of special interest is the electrostatic potential of myosin as determined cataphoretically.[159] Its behaviour is of the ordinary type in salt-free solutions and in the presence of mono monovalent salt; the mobility is rather high on the acid side of the isoelectric point but becomes zero at the isoelectric point at *p*H 5·4 and goes down slowly to negative values at higher *p*H. Yet in the presence of polyvalent cations, especially Ca^{++} and Mg^{++}, the cataphoretic picture is entirely transformed, the molecular potential remaining positive at all *p*H's measured (up to *p*H 9). Concentrations of Ca^{++} and Mg^{++} as low as 0·03 M are sufficient to change the sign of the mobility on the alkaline side from negative to positive. Apparently the combination of divalent cations with the carboxylate groups of the protein takes place according to a mechanism proposed by DANIELLI[160] for protein-Ca combination, and supported recently by DEUEL[161] for the reaction of Ca with pectins, namely

$$RCOO^- + Ca^{++} \overset{K}{\rightleftharpoons} RCOOCa^+$$

This mechanism is evidently different from the cross-linking action of polyvalent ions described previously and plays a great role in regulating the bonding possibilities of myosin by determining its molecular potential.

In addition to the charge-bearing amino acids and the peptide bonds, myosin contains a large number of hydroxyl and sulphydryl amino-acid residues, which provide for effective intermolecular hydrogen bonding. Special importance is attributed to the sulphydryl groups in the formation of the actomyosin complex and in the interaction with ATP. This was specially stressed by BAILEY and PERRY,[162] who demonstrated that oxidation or blocking of the sulphydryl groups prevents actomyosin formation.

The other muscle constituent is the actin isolated by STRAUB.[163] It appears either in the form of isodiametric small molecules, as the globular or G-actin, or in the form of very long molecular fibrils, as the F-actin. G-actin is stable in salt-free solutions at about *p*H 7. Decreasing the *p*H to below 6 or adding neutral salt causes rapid polymerization. The rate of polymerization is markedly dependent on the concentration and nature of the added salt; while monovalent chlorides bring the half time of the polymerization to 15 minutes in ionic strengths of 0·1–0·15, the chlorides of calcium and magnesium cause the same rate in concentration of 10^{-2}–10^{-3} molar. The polymerization of the small molecules of G-actin resembles the fibrillation process described previously for insulin and the serum proteins. It is easily envisaged how the regulation of *p*H and salt concentration determines the interplay of repulsive and attractive forces which govern the rate of fibrillation or what is called in this case "polymerization". Yet in this case seemingly specific stereochemical factors are superimposed, as the polymerization

proceeds in two steps;[164] first, packages 300 Å long and 100 Å thick are formed and, afterwards, these packages unite to form long threads approaching a length of 10·000–50·000 Å.

Among the van der Waals bonding groups, a special role in the polymerization of G-actin is played again by the SH group, so that polymerization is inhibited by agents destroying or blocking this group. Prolonged standing of G-actin diminishes its polymerizing activity, but, remarkably, addition of ATP, which apparently combines with the SH groups by specific van der Waals bonds, protects the actin from deterioration.

Both myosin and actin readily give gels but the most important gels are the products of their interaction—the actomyosins. At ionic strengths higher than 0·2 the actomyosin complex may be kept in solution and its properties investigated. It was found that the complex is of more or less definite proportions in spite of its being formed mainly by van der Waals and electrostatic bonds. Straub found that gradual addition of actin to a myosin solution increases the viscosity till a maximal value is attained at a ratio of 0·4 mg actin to 1 mg myosin.

Special attention has been paid to the influence of ATP on actomyosin solutions. It was found that addition of ATP decreases the viscosity, the flow birefringence, the sedimentation rate, and the light scattering of the solutions. The conclusion drawn by most investigators[158] from these observations is that ATP causes a dissociation of the actomyosin complex. It is assumed that ATP combines with the bonding sulphydryl groups by very strong bonds, which according to Weber may approach 10 Kcal/mole, and thus prevents the actin-myosin combination.

This action of ATP is regarded by these investigators as a plasticizer effect and to it is attributed a special importance in the general effect of ATP on muscle. Yet it should be clearly realized that the conventional approach to the results of various physical measurements when applied to actomyosin may be misleading. The theories of flow phenomena, light scattering, and sedimentation of very long polyelectrolytes are either non-existent or in their infancy, and the assumption that actomyosin behaves as a normal polymer needs careful justification. It is, therefore, impossible to dismiss *a priori* the idea put forward by Jordan and Oster[165] that the phenomena observed are due to the coiling up of the actomyosin molecules under the influence of ATP rather than to their dissociation. This suggestion gains in weight as a result of the recent work of Blum,[166] supported by Morales and Botts,[167] who showed by light scattering measurements, extrapolated according to the method of Zimm,[168] that the molecular weight of actomyosin remains the same whether untreated, or treated by ATP.

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In order to produce optimal conditions for the preparation of gel threads of actomyosin suitable for mechano-chemical experiments, one should reduce the salt concentration to 0·1 m KCl, add 10^{-2}–10^{-3} $MgCl_2$, and keep the protein concentration in the thread between 6 per cent and 7 per cent. Adding to such a thread a fresh extract of muscle containing ATP causes a violent contraction which reduces the length of the thread during the first 30 seconds by 50 per cent and during the next 2–3 minutes by 70 per cent. The first experiments, which were performed on rather poor preparations, caused an overall shrinkage of the threads, both in length and in thickness, which could not be identified with the isochoric contraction of the muscle but was more similar to an ordinary deswelling of an isotropic gel. Yet SZENT-GYORGYI[169] realized early the fundamental identity of shrinking and contraction. Indeed, further experiments proved that better orientation of the molecular network improves the contraction along the orientation axis. H. WEBER[170] was able to prove conclusively that the fundamental process of contraction is identical for glycerol-extracted muscle preparations and for myosin fibres.

The contractile action of ATP should be distinguished from its plasticizer effect, which is common both to ATP and to other polyphosphates. This plasticization by ATP, which apparently keeps the muscle elastic and prevents rigidity, is not very specific and may be simulated by different inorganic salts having no contractile action.[171] The specificity of ATP is more apparent if it is added in the presence of other plasticizers; it causes immediate and strong contraction.

The contraction-releasing action of ATP is related to its splitting by the ATP'ase activity of the native muscle and of the myosin preparations. The brilliant work of ENGELHARDT and LJUBIMOVA[172] on the enzymatic activity of myosin or of some myosin fraction[173] supplies the guiding principle in the understanding of the action of ATP during contraction. When the enzymatic breakdown of ATP is blocked, no contraction occurs. Evidently, the release of the energy stored in the energy-rich phosphate bond is the prerequisite for the muscular activity. Thus, stimulation of the muscle should be connected with the removal of some inhibition which blocks the ATP'ase activity in resting muscle.

In spite of the cardinal discoveries in the field of muscular activity, the final step, in which chemical energy is transformed into mechanical, still remains unknown. The mechano-chemical coupling is still a matter of speculation, for which the pertinent material is derived from thermodynamic studies of the muscle described in the following paragraphs.

Thermodynamic analysis

The applicability of thermodynamic laws to such a kinetic process as the muscular twitch has been much disputed. Nevertheless, it is

doubtless justified for the elastic and thermo-elastic measurement of the resting muscle and it may be used with due caution for the contracted muscle maintained in a state of quasi-equilibrium. Major use has been made of the thermodynamic equation of WIEGAND and SNYDER,[174] which correlates the tensile stress (f), with the inner energy (u), temperature (T), and length (l), of a fibre of constant volume.

$$f = \left(\frac{\partial U}{\partial l}\right)_{T,p} + T\left(\frac{\partial f}{\partial T}\right)_{l,p} \quad \ldots.(40)$$

This equation, which is a straight-forward conclusion of the first and second laws of thermodynamics, is based on a Maxwell relation of the form

$$\left(\frac{\partial f}{\partial T}\right)_{l,p} = -\left(\frac{\partial S}{\partial l}\right)_{T,p} \quad \ldots.(41)$$

where S is the entropy of the fibre.

The great advantage of equation (41) lies in the fact that by a relatively simple measurement of the stress (f), and its dependence on the temperature at isometric conditions $\left(\frac{\partial f}{\partial T}\right)_{p,l}$, we get simultaneously the entropy and inner-energy changes accompanying stretching.

On *a priori* grounds, it might be expected that in an ideal rubber whose elasticity is founded on the thermal, random movement of molecular segments, the resistance to stress would be determined by the decrease in configurational entropy accompanying the transition from randomness to a certain degree of order. No change in inner energy should result from the stress so that $\left(\frac{\partial U}{\partial l}\right)_{p,T} \sim 0$. On the other hand, in a steel spring where the crystalline arrangement keeps the iron atoms in fixed positions, the main resistance to stretch is due to the increase in the interatomic inner energy so that $\left(\frac{\partial U}{\partial l}\right)_{p,T}$ should be large and positive, while the entropy contribution is negligible in comparison.

Highly stretched rubber, in which local crystallization occurs, presents new features. The crystallization process orients the macromolecules even more than mechanical stress so that a large decrease in the entropy factor $T\left(\frac{\partial f}{\partial T}\right)_{p,l}$ should occur. Simultaneously, crystallization heat is liberated, making the change in the inner energy negative and large. In fully stretched rubber, we finally approach the behaviour of a steel spring: the macromolecules are stretched and oriented so that the entropy contribution becomes negligible while the resistance to stretch is determined by the positive increase in the inner

energy of the interatomic bonds. The expected behaviour is very clearly demonstrated in Fig. 20 of GUTH.[175]

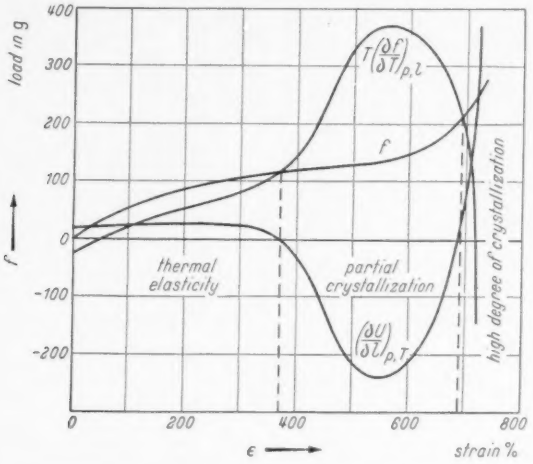

Fig. 20. Resolution of the stress (f) for natural rubber into entropy, $T\left(\frac{\partial f}{\partial T}\right)_{p,l}$ and inner energy $\left(\frac{\partial U}{\partial l}\right)_{p,T}$ (from GUTH, E.; *Ann. N.Y. Acad. Sci.* 47 (1947) 723)

If we now compare the stress-strain relations of a resting muscle with that of rubber, it is found that the picture corresponds to that of highly stretched rubber in an advanced degree of crystallization (Fig. 21).

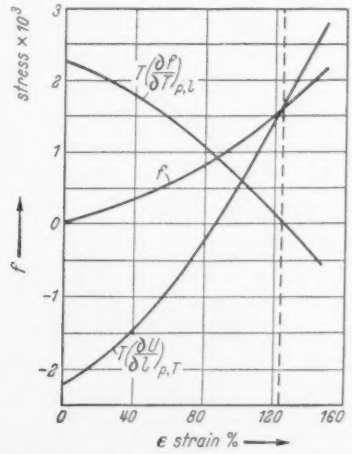

Fig. 21. Resolution of the stress (f) into entropy and inner energy components for *ligamentum nuchae* (from GUTH, E.; *Ann. N.Y. Acad. Sci.* 47 (1947) 743)

These data for relaxed muscle prove convincingly that muscle is a highly organized structure, as may also be inferred from its double

refraction and diffraction pattern.[176] As the experiments of RAMSEY and STREET[177] cast some doubt on whether the thermoelastic experiments with whole muscle represent the behaviour of the contractile actomyosin apparatus or of the exterior sarcolemma, it is of interest to note that isolated fibre models exhibit the same behaviour as the whole muscle.

A. and H. H. WEBER[178] determined the thermal coefficient of isometric stress

$$\beta = \frac{l}{f}\left(\frac{\partial f}{\partial T}\right)_{p,} \qquad \ldots.(42)$$

in isolated fibres and found it to be appreciably larger than $\frac{l}{T}$. These measurements indicate that $\left(\frac{\partial U}{\partial l}\right)$ is negative and large, as according to the equation of WIEGAND and SNYDER

$$\beta = \frac{l}{T}\left(l - \frac{l}{f}\left(\frac{\partial U}{\partial l}\right)_{p,\,T}\right) \qquad \ldots.(43)$$

so that for $\beta > \frac{l}{T}$, $\left(\frac{\partial U}{\partial l}\right)_{p,\,T} < 0$, f being positive.

Additional evidence for the generality of the conclusion about the orderly and presumably crystalline arrangement is found in the measurements of BOTTS,[67] who found that in actomyosin threads, just as in whole muscle, both the entropy and the inner energy change during contraction.

Although the results seem to be well established, the conclusion drawn by most writers that the crystallization which accompanies stretch is the only possible source of decrease in free energy, is not convincing. It should be clear that the actomyosin molecules are charged and characterized by a repulsive energy inversely proportional to their end-to-end distance according to equation (18). Stretching the molecules decreases the repulsion of the fixed charges and reduces the inner energy. Thus, although the presence of crystallization forces in resting muscle is very probable, the fuller analysis of the change in the inner energy would require the consideration of two factors: the decrease due to attractive forces and that due to the diminution of the repulsive electrostatic energy.

The next step is to investigate the elastic behaviour of the contracted muscle in the state of activation. Distinguishing traits are the diminution of double refraction, and the blurring of the X-ray diagram which accompanies contraction.[179] This would indicate that the order of the resting muscle decreases and we pass into the range of more random rubberlike behaviour. That the optical phenomena are the result of true disorientation and not of the increase of tension in the activated

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muscle may be deduced from the observations of VON MURALT and EBNER[180] that at isometric conditions the increase in tension does not alter the birefringence. Of special interest for the understanding of the contracted state are the new measurements of PRYOR[181] on the dynamic rigidity modulus of muscle. PRYOR determined the amplitude of a vibrating muscle versus frequency and from the measurements obtained a conclusive proof that the entropy contribution of the muscle elasticity increases markedly with active contraction. He interprets these results in a manner similar to the theory of collagen shrinkage discussed above. According to PRYOR, ATP acts as a plasticizer which penetrates the muscle crystallites and liberates the long-chain molecules, which subsequently contract with gain in configurational entropy. This attractive theory, which co-ordinates many known facts in a single framework, has, however, been criticized on various grounds. First, plasticizing action *per se*, as pointed out previously, is insufficient to induce contraction and only with the specific enzymatic splitting of ATP may contraction take place. Secondly, if the ATP acts as a plasticizer, it should lower the melting point of actomyosin. According to WEBER, this is not borne out by actual experiments.

Various other theories have been advanced to explain the nature of contraction. Well known is the spiral model of SZENT-GYORGYI,[169] which is based on the assumption, further elaborated by ASTBURY,[182] that the actin depolymerizes during contraction. We should like only to emphasize a group of theories which attribute a special role to the electrostatic forces in the muscle fibre. K. H. MEYER[183] suggested that the resting muscle is stretched by the net charge of the myosin molecule. Contraction results from the strong attraction between the balanced charges of the carboxylate and ammonium groups. But as the *p*H of the muscle is practically constant, this theory fails to propose a factor which might influence the balance and change the net charge of the myosin molecules.

RISEMAN and KIRKWOOD[184] suggested that the stretching agents are phosphate groups introduced into the myosin by ATP in the relaxation step. A more general treatment of the phenomena has been proposed by KATCHALSKY[119] and by KUHN[185] for polyelectrolyte systems, which will be presented subsequently. Recently, MORALES and BOTTS[167] proposed a new modification of the theory based on the reviewer's ideas. They suggest that the specific adsorption of ATP is the electrostatic factor which was lacking in MEYER'S original suggestion. Their theory is based on the following observations: 1. ATP is strongly and specifically adsorbed by forces in addition to coulombic; 2. The adsorbed ATP bears a high net charge—4 negative charges at *p*H 7; 3. It is enzymatically decomposed by myosin into non-adsorbed ions (ADP and orthophosphate) of much lower free energy. The effect of added ATP

should depend on initial circumstances. If the myosin were positively charged and a moderate amount of ATP added, the absolute value of the net charge of the particles would decrease and the myosin should contract. If the myosin were negatively charged and ATP were added, the absolute value of the net charge would increase and the myosin particles should extend. In all cases, the effect of adding ATP should be spontaneously reversible—rapidly if the state of the system favours ATP'ase activity, and slowly if it does not. The theory is as yet unable to give a detailed account of the contraction process and the exact nature of the complex of operative forces, but it adds explicitly to the hydrogen-bond-forces and crystallization tendency, the electrostatic factors.

In summary, it may be stated that most of the investigations contain partial truths: crystallization and plasticization, hydrogen bonds and electrostatic forces, thermoelastic rubberlike contractility, all of them play a role in the complex living phenomena, but their relative importance in the mechano-chemical transformation in the muscle awaits further elucidation. Yet out of the concentrated effort to solve the problems of animate motility emerges slowly another and more general concept, that of mechanisms which may transform chemical into mechanical energy in isothermal cycles. The abstract study of mechano-chemistry as a branch of statistical thermodynamics, based on experiments with simple synthetic models, may also help in the elucidation of living motility.

Polyelectrolyte models of mechano-chemical systems—First steps in the development of synthetic contractile systems have been made during recent studies of polyelectrolyte gels. The realization that ionized macromolecules in solution are capable of reversible contraction and extension[119] as a result of intramolecular electrostatic forces suggested the possibility of amplifying the effects on a macroscopic scale. Three laboratories observed simultaneously[19a, b, c] that cross-linked polyacrylic- and polymethacrylic-acid gels are capable of rapid cyclic expansion and shrinkage by successive additions of alkali and acid. The extent of the change may be read directly from the swelling isotherms at different degrees of ionization given in Fig. 12. Very spectacular were the experiments on threads of polyvinyl phosphate:[20] on addition of strong alkali the threads expanded to about three times their original length, while the addition of mineral acid caused rapid reversion to the original length.

It was found in all cases that the mechanical strength of the synthetic models increases markedly with the degree of cross-linking, so that threads of low percentage of cross-links were too weak to give repeated mechanical performance. However, highly cross-linked threads, though stronger mechanically, give smaller contractions and expansions, as

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might be predicted theoretically from equation (24). W. KUHN and B. HARGITAY[185] prepared strong films of 80 per cent polyvinyl-alcohol and 20 per cent polyacrylic acid suitable for mechanical experiments. The films were vulcanized under stress at 80°C to give insoluble gels of pronounced double refraction. Their mechanical strength permits strips of the material to carry loads of many times their own weights. A strip carrying an attached weight, if dipped

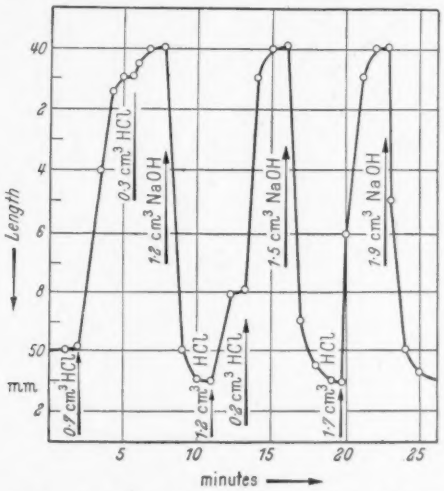

Fig. 22. Cyclic changes in length of a synthetic model of 80 per cent polyvinyl alcohol and 20 per cent polyacrylic acid, due to successive additions of acid and alkali. The exterior medium contains 0·01 N NaCl. The added HCl and NaOH are both 0·02 *n*. The weight of the fibre is 6 mg, carrying a load of 360 mg (from KUHN, W. and HARGITAY, B.; *Experientia* 7 (1951) 1)

alternately into solutions of alkali and acid, will expand and contract, performing mechanical work at the expense of the energy of neutralization. Fig. 22 represents one of KUHN and HARGITAY's experiments with cyclic additions of sodium hydroxide and hydrochloric acid. The mechanical reversibility of these systems is good and the experiments may be repeated many times without fatiguing the system.

Special attention has been paid to the "efficiency" of the synthetic models and to their technological potentialities. As the gel is made to work in cycles, its chemical participation in the work cycle cancels out, so that in the contraction experiments described above the supply of the chemical energy is due only to the reaction $NaOH + HCl \rightarrow NaCl + H_2O$. This reaction is highly exergonic ($\Delta F = -17$ Kcal/mole) and, as may be deduced from Fig. 22, only a very small fraction of its free energy is transformed into mechanical work. KUHN and HARGITAY proposed a cycle in which the gel may perform work reversibly when coupled with suitable osmotic devices. In this cycle, the free energy of

neutralization may be transformed in full into mechanical work which is performed partly by the auxiliary osmotic devices and partly by the gel itself. The authors attribute an efficiency of 100 per cent to this cycle. Evidently, free chemical energy of any reversible reaction is converted into available mechanical energy with 100 per cent efficiency; however, in the case under consideration, it is only that part of the

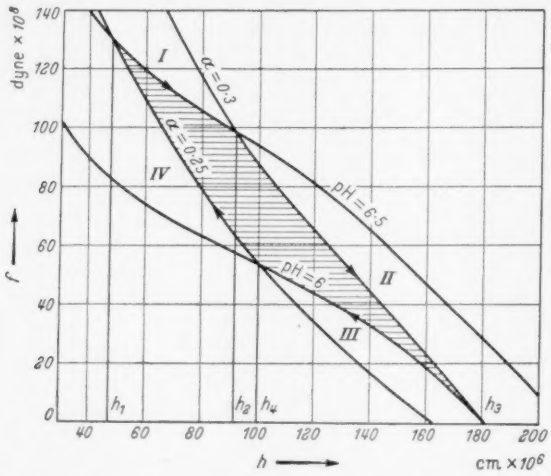

Fig. 23. Mechano-chemical cycle of a single polymethacrylic acid molecule ($Z = 10{\cdot}000$) working between two states of ionization [$\alpha = 0{\cdot}3$ and $\alpha = 0{\cdot}25$] and two pH values (pH 6·5 and pH 6) at constant temperature (from KATCHALSKY, A.; *J. Polymer Sci.* 7 (1951) 393)

work which is performed by the gel itself which should be regarded as the efficiency of the gel machine.

A mechano-chemical reversible cycle which does not require osmotic devices was described by KATCHALSKY.[119] The gel under consideration consists of fully oriented polyelectrolyte molecules with all their end-to-end distance vectors (h) lying parallel. Such a gel may be treated as a multiple of simple single-molecule engines. The essential features of such a gel-machine are: 1. Easy maintenance of equilibrium with chemical reservoirs containing the reactant agents, and 2. The possibility of changing the potential of the bound reactant through the extension or contraction of the gel while isolated from the reservoir.

The complete work cycle depicted in Fig. 23 comprises four steps: two "isopotential" steps in which the gel is stretched (I) or contracted (III) while in equilibrium contact with two buffer solutions of pH 6·5 and pH 6, respectively; and two "isophoric" steps (II and IV) in which the gel, being isolated from the chemical reservoirs, performs work without going into reaction with the buffer, and therefore maintains a constant charge.

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In the isophoric step the electrostatic potential does not remain constant: e.g. stretching the molecules diminishes the potential and thereby the pH drops according to the potentiometric equation (36). Thus, the isophoric steps permit the reversible transition from a bath of one pH to a bath of another pH. On the other hand, stretching the gel isopotentially while in equilibrium with a buffer solution of constant pH, results in chemical reaction which maintains the molecular potential constant but increases the degrees of ionization α.

The shaded area in Fig. 23 represents the useful work which is performed by the gel-machine in transferring chemical neutralization energy from a higher potential at pH 6·5 to a lower one at pH 6. Clearly, the whole of the transferred chemical free energy is transformed into mechanical energy. We, therefore, see that reversible mechano-chemical engines which operate only by means of network deformations are possible theoretically and may be realized experimentally.

Mechano-chemical engines may vary widely according to the nature of the chemical reactions involved, and to the chemical-potential difference of the two isopotentials of the cycle. The potential difference which may be utilized mechano-chemically, is determined by the ability of an isophor to join two distant isopotentials. Randomly-linked macromolecules, operating only by means of electrostatic forces, undergo only a very limited change of chemical potential during an isophoric process, and hence may not develop into devices of practical value. However, the introduction of additional factors may change profoundly the shape of the isopotentials and isophors, and widen the area of the work cycles. Almost all the factors discussed throughout this review: reversible cross-linking by polyvalent ions, crystallization forces, hydrogen bond attraction and specific interaction with suitable reagents, may participate in shaping the mechano-chemical mode of operation. It is the aim of further investigation to co-ordinate the different factors into a unified framework capable of providing an interpretation of existing data and of predicting new working possibilities.

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2

THE TRANSFERENCE OF THE MUSCLE ENERGY IN THE CONTRACTION CYCLE*

H. H. Weber and Hildegard Portzehl

A. Introduction

It is characteristic of Otto Meyerhof that he should have answered one of the fundamental biological questions before the majority of the biologists had even asked it. The question is this: How does it come about that all living organisms are capable of utilizing equally well—i.e. with nearly the same efficiency—the energy of such very different substances as protein, carbohydrate and fat for many different and highly specific functions? The fact that the organisms are capable of this is already implied by Rubner's "isodynamic law". The way in which the organisms accomplish it, however, is explained in principle by Meyerhof as follows: In every case, a specific operative substance is required for a specific function. This substance exists only in small quantities but, as a rule, does not substantially diminish during the performance of the work, as it is at once restored. This occurs in a sequence of cyclic reactions which are partly consecutive and partly parallel, but which are chemically and energetically coupled with each other. The further these restoring cycles lie behind the primary working process the more energy do they supply. It may be supposed that:

1. In the more remote cycles a turnover takes place of those substances which exist in ample quantities.

2. In the later stages of recovery several cycles run in parallel, so that different energy-liberating processes are at the same time available and can be used simultaneously or in turn. Thus, in general, during the anaerobic recovery period, the whole of the energy supplied from the fermentation of carbohydrate is ready for use, whilst the oxidative recovery yields the energy released by the oxydation of carbohydrate, fat and protein in addition.

It was discovered by Meyerhof and his pupils (Lohmann[112]) in connection with the work of Hill, Parnas, Embden, and Eggleton and Eggleton[49] as well as that of Fiske and Subbarow,[56] that the adenosine triphosphate (ATP) cycle lies at the beginning of the chain of linked cycles. The part of this chain of reactions definitely recognized and ascertained by the schools of Meyerhof, Warburg, and Cori ends, at present, with the anaerobic part of the carbohydrate cycle.†

* Translated from the German by Marga Weber, Tübingen.

† See the reviews of Lohmann,[113] v. Muralt,[133] Meyerhof,[121], [122], [123] Hill,[82] D. Needham,[134] Lehnartz[110] and Parnas.[136], [137]

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One of the most urgent questions in biochemistry is by what special reactions the energy set free by oxidation restores the anaerobic processes of decomposition. For twenty years a second open question has been whether the breakdown of ATP is actually the first link in the chain of energy-releasing reactions, or whether the energy liberated by the splitting of ATP serves only to restore another substance—the still unknown immediately operative substance. This was one of the most important questions in the field of muscle physiology. So long as it remained unanswered it could not be decided whether the energy necessary for the working cycle of the muscle is provided during the phase of contraction or during the phase of relaxation.

The situation is now changed: we know now that the breakdown of ATP is the immediate source of energy of the working cycle. Further, the energy set free by the splitting of ATP is transmitted during contraction and not during relaxation and the ATP is not only the source of energy of contraction, but is also the shortening agent.

We do not know, however, by what molecular mechanism ATP and the splitting of ATP brings about the change in the shape of the contractile actomyosin particles.

The contraction of the living muscle is not the initial link but an intermediate one in the chain of reactions of the working muscle. The chain begins in the living muscle with those processes of the muscular membrane (depolarization, new polarization with opposite charge, and the reversal of both) of which the wave of excitation is composed. Thereupon a process of transmission proceeds from every point of the excited membrane into the depths of the muscular fibre right down to the innermost contractile fibril—and with a speed much greater than the highest speed of diffusion of any substance in a watery solution (A. V. HILL[88] according to statements of B. KATZ).

A few milliseconds later an explosive production of heat throughout the entire fibril cross-section occurs (A. V. HILL[93]). Simultaneously a change in the state of the contractile protein takes place, which is recognizable by changes in the diffraction (D. K. HILL[95]), in the elastic properties (A. V. HILL[91], [92], [93]) as well as by the phenomenon of latency relaxation of the taut muscle (RAUH[153], SANDOW[158] ABBOTT and RITCHIE[5]). Just a few milliseconds later contraction begins.

These features of muscular contraction give rise to a *second* problem, i.e. to define the limit between the working cycle and the *preceding* links of the chain of reactions. At the same time one is prompted to ask: how are the two mechanisms coupled? The first problem can now be considered as solved. As to the nature of the connection between the processes of excitation and contraction certain conclusions can be drawn.

For it is possible to isolate the working cycle experimentally thereby

separating it from the processes of excitation and transmission on the one hand and of recovery on the other hand. In the presence of the Marsh-Bendall-factor (M-B-factor) (see below) small changes in the nature and concentration of the ions present can determine whether the contraction of the isolated working cycle sets in or is inhibited. Thus, the migration of ions during the process of excitation may control the behaviour of the isolated working cycle. Further, it is possible to separate experimentally the process of contraction from that of relaxation, thus enabling a separate investigation of the conditions of both processes.

This isolation is achieved by progressively breaking up the whole muscle into simpler components. In the first stage the whole structure of fibrous proteins is kept intact and the M-B-factor, also obtained from the muscle, is added (fibre model + M-B-factor). The second stage makes use of the fibre model without M-B-factor. The third stage is an oriented system of pure actomyosin, the contractile protein of the muscle (thread model). In the fourth stage the actomyosin gel is unoriented.

The present review will deal with the properties of these systems and their behaviour on the addition of ATP and other polyorthophosphates.

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B. The Conformity of the Working Cycle of Different Kinds of Muscles in Vivo and Isolated

1. The isolation of the working cycle

(*a*) *The different kinds of models*

In 1932 the oriented (acto-) myosin thread was introduced as a model which could help to explain the mechanical properties, x-ray-diffraction, and the birefringence of the muscle (Boehm and H. H. Weber[24] and H. H. Weber[195], [196]).

In 1939 Engelhardt and Ljubimova[50] found that these threads split adenosinetriphosphate (ATP) and thereby become more extensible. This effect of ATP will henceforth be called "plasticizing" effect.

In 1942 Szent Györgyi[172] observed that the actomyosin thread shrinks enormously on the addition of ATP at *p*H 7·0 (if the thread has not been dried and therefore is unoriented (Szent Györgyi[172])). At the same time, Gerendas[62] reported that the thread could be oriented by stretching without the necessity of drying if certain metal ions were added. However, the capacity of the threads to react to the addition of ATP was completely (Cu) or largely (Zn) destroyed. The residual activity of ATP seemed to give rise not to an isodimensional shrinkage but only to an actual shortening. Szent Györgyi and his school ever since have called the shrinkage of these threads a "contraction". In

1947 BUCHTHAL[33] and co-workers succeeded in making actomyosin threads extensible by partially drying them and they were thus able to orient them to some extent without additions of any other substances. When unloaded, these threads showed an actual shortening on immersion in a solution of ATP (2×10^{-3} M). But after loading an increase in length was observed, which under some conditions was quite considerable (BUCHTHAL, DEUTSCH, KNAPPEIS and PETERSEN[33]). Thereupon BUCHTHAL was disinclined to recognize a correlation between "contraction" in actomyosin threads and the contraction of muscle (similarly also PERRY, REED, ASTBURY and SPARK,[140] JACOB,[97] SANDOW,[159] ASTBURY[7]).

In the meantime VARGA[186] (in SZENT GYÖRGYI's laboratory) had prepared microtome sections of muscles, about 100 μ thick, which after extraction with water and freezing, shortened to 30 per cent of the original length when placed in a bath of ATP. It was not possible to measure the tension they developed, because they tore too easily.

In 1949 SZENT GYÖRGYI[174] substituted for the microtome sections bundles of fibres 0·2 to 0·5 mm in diameter from the psoas muscle of the rabbit, and found that these fibre bundles developed tensions of up to 2 kg/cm^2 on addition of ATP.

In 1950 it became possible to prepare oriented actomyosin threads which contract with development of high tensions and increase in thickness (PORTZEHL and H. H. WEBER,[148] PORTZEHL[144]). As a second model for contraction the *single* fibre, extracted with glycerol and water, was introduced (A. WEBER and H. H. WEBER,[193], [194] A. WEBER[192]). Threads of actomyosin and single extracted fibres were taken which were so thin that unsplit ATP could reach the centre of the cross-section in steady state diffusion. With these models, in contrast to the former preparations, the working cycle of the living muscle is so accurately imitated that no doubt can be entertained as to the isolation of the almost unaltered working cycle. At the same time the conditions for contraction are much more sharply defined than with former models.

In 1952 a still closer similarity to the physiological state was achieved by the addition of the M-B-factor to the glycerol-water extracted muscle fibre (BENDALL[18]).

All these preparations are not only capable of contracting but also of relaxing (C Ia, b and C V).* But this does not apply to another type of model contraction which occurs if a frozen muscle is thawed. This type has long been known (DUBOIS-REYMOND,[47] KÜHNE,[103] HERMANN[80]). In 1949 it was explained by SZENT GYÖRGYI as an ATP-induced contraction, caused by the ATP which has remained in the muscle itself (SZENT GYÖRGYI[174] as well as BORBIRO and SZENT GYÖRGYI,[25] CREPAX and HERION,[39] CREPAX,[38] GODEAUX[65]).

* Pages 82 and 101.

Finally, the shrinkage of unoriented actomyosin gel and that of precipitate (super-precipitation (SZENT GYÖRGYI)) occurring on the addition of ATP have both been shown to be due to the same experimental conditions as the fully developed contraction of the oriented actomyosin threads and fibres. Accordingly super-precipitation and shrinkage are a technically simple means of testing qualitatively the factors which influence contraction (SZENT GYÖRGYI,[176] H. H. WEBER and PORTZEHL,[204], [205] KUSCHINSKY and TURBA[105]).

(*b*) *Preparation and general properties of the models*

In order to obtain correct results, the extracted muscle fibre and the actomyosin thread must not be too thick. For, in a stationary state of diffusion, the concentration of ATP (C) decreases from the outer layers of the model to the centre, owing to the hydrolysis of ATP, according to the Meyerhof formula (MEYERHOF and SCHULZ[124]):

$$C = \frac{Ar^2}{4D} + J$$

(A = rate of splitting, r = radius of the cylinder, D = diffusion constant.) In this equation the first term indicates the decrease of the ATP concentration along the diffusion path from the bath to the centre of the model and J the concentration at the centre when the concentration outside the model is higher than the decrease of concentration along the diffusion path $\left(C > \frac{Ar^2}{4D}\right)$. In the opposite case, a core of the cylinder model remains free from ATP (A. WEBER,[192] H. H. WEBER[200]). This core falsifies the phenomena of contraction at least quantitatively, as it does not participate in contraction and is also "rigid" (see Section B, II). This is always true of fibre bundles.

MEYERHOF's formula is valid only if the rate of splitting A is independent of the concentration. In this case, the lower the concentration in the bath, C, the smaller r must be to avoid C lagging behind $\frac{Ar^2}{4D}$. As the rate of ATP breakdown does not in fact decrease when the concentration of ATP is reduced to 2×10^{-4} M (HASSELBACH[73]), it is evident that the Warburg-Meyerhof *Grenzschichtdicke* (limit of the permissible thickness) is considerably altered with the concentration of ATP.

The *Grenzschichtdicke* of the fibre model can be accurately calculated on the basis of HASSELBACH'S[72], [74] studies. The diffusion constant inside the model fibre is $2\text{–}3 \times 10^{-8}$. (This value is obtained by measurements of the dependence of the rate of splitting of ATP on the thickness of glycerol-water-extracted slices of muscles.) The diffusion constant of ATP is about 100 times smaller inside the fibre

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than in free solution, viz., 4×10^{-6}, (A. WEBER[192]). This value and the hydrolysis rate inside the fibre model determine the upper limit of the thickness of the fibre corresponding to every concentration of ATP. In a physiological concentration of about 5×10^{-3} M, the diameter of the fibre of skeletal muscle must not exceed 30 μ.

A diameter of approximately 60 μ seems to be permissible both for fibre models prepared from smooth muscle (G. and M. ULBRECHT[184]) and for actomyosin threads (PORTZEHL[144]), owing to the smaller rate of splitting (A) and loose structure of these preparations.

In order to make actomyosin threads contract with the development of tension, the protein concentration must be regulated; if the concentration is too low, the cohesive forces will be too small; if too high, the movement of the protein particles will be too restricted. The optimal concentration of protein lies between 6 to 10 per cent, but the concentration is as low as 1 to 2·5 per cent, when the thread is freshly extruded. If the thread is dried in air for a few minutes (see BUCHTHAL *et al.*[33]) the increase in protein concentration is much greater near the periphery than in the centre, and this is also true of the orientation and birefringence which develop on stretching. This inhomogeneity caused by drying can be avoided by first soaking the thread in a glycerol-water mixture and then drying it at 1°C in an atmosphere almost saturated with water vapour. In this way, the drying process takes many hours, and the centre and periphery now remain in water equilibrium. The final protein concentration is determined by the proportion of glycerol in the glycerol-water mixture, and cannot exceed the desired concentration anywhere in the fibre (PORTZEHL and H. H. WEBER,[148] PORTZEHL[144]).

All models denature if kept in a watery solution for hours. In glycerol (density 1·23) the working capacity of fibre models prepared from the smooth posterior adductor muscle of *Anodonta* greatly diminishes in a few days even at -9°C (G. and M. ULBRECHT[184]). Fibre models from the psoas muscle of rabbit can be kept in glycerol at 0°C for about a fortnight; threads prepared from the actomyosin of rabbit can be kept several months without showing a noticeable decrease of their working capacity (A. WEBER,[191] PORTZEHL[142]).

Extraction of muscle fibres with glycerol-water mixtures removes 20 per cent of the muscle protein—especially proteins of albumin character (SZENT GYÖRGYI[174]). The proportion of actomyosin is thereby increased from a value of about 50 per cent in the case of living muscle (HASSELBACH and SCHNEIDER[76]) to one of about 65 per cent. Of the crystalloids originally present, only a few cations, mostly K, remain behind as *Gegenionen* of proteins, and these are mostly replaced by Na ions when the extracting solution is buffered with Na salts (HARRIS[71]).

Threads prepared from actomyosin which has been repeatedly precipitated contain no other protein. The concentration of the crystalloids depends only on the composition of the bath—not taking into account traces of Mg which are strongly bound by actomyosin (see C, V).

Consequently, the contractile properties of the models which are common both to the extracted muscle fibre (fibre model) and to the actomyosin thread (thread model) can be ascribed to the actomyosin.

A quite new preparation of oriented actomyosin threads rich in protein has been made from monolayers in the LANGMUIR trough (HAYASHI[77]). It has long been known that actomyosin forms monomolecular layers on neutral watery solutions of salts (MOSS and RIDEAL,[128] DERVICHIAN,[41] MUNCH-PETERSEN[131]). When such films are excessively compressed they collapse into stripes, in which the individual particles are mostly orientated parallel to the long axis of the stripes. In spite of the denaturation of the monolayers the stripes contract, with development of tension, to a maximal value of 50 per cent of the initial length.

II. The elastic state of muscle and models in rest, rigor and contraction

(*a*) *Resistance to stretch of the contractile structure in the states of rigor and rest*

It is certain that the resistance to stretch of the skeletal muscle is preponderantly determined by the resistance to stretch of the contractile substance itself, as long as the length of the muscle does not exceed or only very slightly exceeds its equilibrium length. If the muscle is stretched much beyond the equilibrium length, the elastic resistance of the sarcolemma and the connective tissue becomes more and more obvious (RAMSEY and STREET,[151], [152] BUCHTHAL and KAISER,[34], [35] A. V. HILL,[86], [90] CASELLA,[36] MAURO and STEN-KNUDSEN[127]).*

As a result, the resistance to stretch exerted by the contractile substance corresponds to that of the whole muscle only in the following conditions: (*a*) if the muscle, before the measurement of the resistance, has been brought into a shortened relaxed initial state by stimulation without a load, (*b*) if it is in a state of contraction or contracture, and (*c*) if it is only stretched slightly beyond the equilibrium length ($\sim 0{\cdot}8$ of the resting length *in situ*).

It then becomes evident that the resistance to stretch of the skeletal muscle in rigor is at least ten times larger than that of the resting

* Divergence of opinion exists, however, among the authors mentioned as to the degree of stretching at which the elastic resistance of the sarcolemma becomes noticeable, and how much of the total resistance of a highly stretched muscle is accounted for by this factor.

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living skeletal muscle (see Tables 1 and 2). It does not matter how the rigor has been produced (Table 1) and whether or not it is accompanied by shortening (contracture) (BATE-SMITH and BENDALL[15], [16]).

TABLE 1

Extensibility and ATP-content of muscle in rigor compared with corresponding properties of fresh muscles

Rigidity induced by	*Extensibility rigor/normal*	*ATP-content rigor/normal*	*Author*
Rigor mortis	1/10	1/10	BATE-SMITH and BENDALL[15], [16]
			ERDÖS[51]
		2/10	BENDALL[17]
Thawing (after freezing)	1/10	1/10	CREPAX and HERION[39]
Chloroform	1/10	1/10	ERDÖS[51]
Fluoride	—	1/10	GODEAUX[66]
Monohalogenacetate and stimulation	1/10	1/10	ERDÖS[51]

On the other hand, a complete or nearly complete disappearance of ATP or phosphocreatine in the muscle seems to be a prerequisite for the onset of rigor (Table 1). Especially characteristic is the fact that monohalogen-acetate and the other thioloprive agents lead to rigor only when care has been taken to stimulate the muscle beforehand, so that the vital ATP and creatine phosphate content has been consumed. Thioloprive agents initiate a very rapid decomposition both of ATP and creatine phosphate, as they block the restoring cycle of carbohydrates (GODEAUX[66]).

Since the decomposition of ATP and creatine phosphate are the only metabolic processes common to the different types of rigor, it is clear that the normally high extensibility of the resting living muscle is dependent on an adequate concentration either of ATP or of creatine phosphate.

On the following grounds it is concluded that ATP is the necessary factor, and not creatine phosphate:

1. BENDALL[17] has shown that after death creatine phosphate is largely or completely broken down without an increasing resistance to stretch. Rigor mortis does not set in until after ATP, too, has disappeared.

2. From the behaviour of the muscle fibre extracted with glycerol-water; without ATP and without creatine phosphate the fibre model

TABLE 2

Resistance to stretch of skeletal muscle and models with ATP and without ATP (PORTZEHL[146])

1	2	3	4	5	6	7	8	9	10
Experimental group	*Preparation*	*State*	*Temp.* (°C)	$L/L_0 \cdot 100$	*Resistance to stretch in* $gcm^{-2} \times L \times \Delta L^{-1}$		*Column 6 / Column 7*	*Numbers of Experiments*	*Author*
					with ATP	*without ATP*			
1	Rabbit psoas	Resting	20	100	610	—	0·12		Bate-Smith and Bendall[15], [16]
		Rigor mortis	20	100	—	5000			
		Resting	20	100	900	—	0·18		
		Rigor mortis	20	100	—	4900			
		Resting	20	100	890	—	0·12		
		Rigor mortis	20	100	—	7400			
	Frog sartorius	Resting	20	100	100	—	—		Wöhlisch and Clamann[208] Buchthal[30]
			20	100	150	—			
			—	100	500	—			
Average	*Intact muscle*	*Resting*	—	*100*	*500*	—	*0·14*		
		Rigor mortis			—	*6000*			
2*	Fibre model	Resting	3	90	380	—	0·06	5	Portzehl[146]
		Rigor	3	90	—	7000		3	
3*		Resting	20	50	600	—	0·13	4	
		Rigor	20	50	—	4600		4	
4†		Resting	1	70	680	—	0·08	2	
		Rigor	1	70	—	8350		2	
Average	*Fibre model*	*Resting*	—	—	*~ 520*	—	*0·08*	*11*	
		Rigor			—	*~ 6200*		*9*	

* Rigor: 10^{-2} M phosphate *p*H 7 + 10^{-3} M $MgCl_2$.
Resting: the same mixture + 3×10^{-3} M ATP + 4×10^{-4} M salyrgan = salicyl-(λ-hydroxymercuri-β-methoxypropyl)amide-O-acetate.

† Contraction is nearly completely inhibited by lack of Mg^{++} (the fibres are washed out with Graham's salt before the experiment) combined with low temperatures (0°); bath: 10^{-2} M phosphate *p*H 7 + 7×10^{-3} M ATP.
Rigor: the same mixture but without ATP.

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from the psoas muscle of the rabbit has the same high modulus of elasticity as the psoas muscle in rigor (Table 2, see column 7). Without creatine phosphate but with an ATP concentration of 7×10^{-3} M it has a 10 times smaller modulus, i.e., the same modulus as the resting living muscle (Table 2, column 6). To demonstrate this, activation by ATP must be completely inhibited or considerably weakened. This

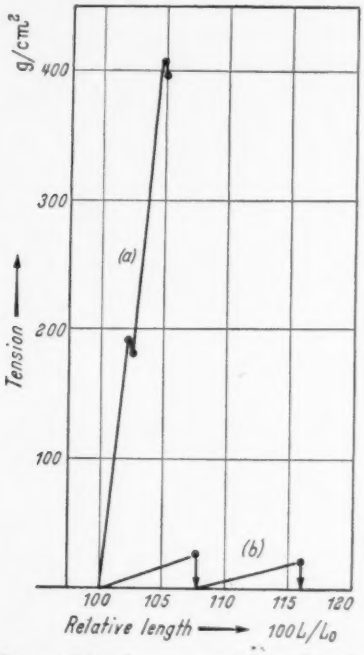

Fig. 1. The dependence of tension on stretch under the influence of the M-B-factor and ATP (HASSELBACH[75]). (Temperature 20°C, ionic strength 0·11 μ, 7×10^{-3} M Mg^{++}, *p*H 7, fibre model + M-B-factor). (*a*) without ATP, (*b*) with 6×10^{-3} M ATP. The fibre is stretched in 2 steps. The arrows indicate the decrease of tension following each step of stretch within 2 min. (Relative length 100 L/L_0 where L_0 is the equilibrium length without ATP. Equilibrium length 0·7 to 0·8 × length *in situ*)

can be done either by adding salyrgan* or the M-B-factor, or by working at a low temperature and in the absence of magnesium (experimental groups 2–4 of Table 2).

If contraction by ATP is completely prevented and denaturation avoided (by salyrgan at 0°C or by the M-B-factor at any temperature) the fibre model is rendered extensively plastic. This is the case when a physiological ATP concentration of 5 to 10×10^{-3} M is added (Fig. 1; in the experiments in Table 2 only 3×10^{-3} M ATP has been used in general). This behaviour of the fibre model is in accordance with

* Salyrgan = Salicyl-(λ-hydroxymercuri-β-metoxypropyl)amide-o-acetate.

HILL's observations of the plasticity of the resting muscle which has been previously shortened by stimulation without load (A. V. HILL[87], [90]).

The fact that ATP acts as a plasticizing agent on actomyosin had already been discovered by ENGELHARDT and LJUBIMOVA[50] as mentioned above. It has been confirmed repeatedly by SZENT GYÖRGYI[174], [176]. The results in Table 2 (PORTZEHL[146]) show, in addition, that ATP seems to be the *only physiological* plasticizing agent in the skeletal muscle. To what extent the smooth muscle and its models become rigid for lack of ATP is not yet known.

(*b*) *The resistance to stretch of the contractile structure during contraction*

The question of whether the modulus of elasticity of the living skeletal muscle during contraction is greater than at rest has been much discussed (GASSER and HILL,[61] STEINHAUSEN,[167] BUCHTHAL,[30] A. V. HILL,[86], [91] BUCHTHAL and KAISER,[35] REICHEL[155]). The discussions, on the whole, are not so much discussions of experimental observations as of the definition of the correct modulus of elasticity in the state of contraction; for the modulus of the resting skeletal muscle depends on its state of stretching, i.e. on its relative length and tension. On the other hand, it is only possible to compare the resistance to stretching exerted by resting, as well as contracted, skeletal muscle when they are either at equal length or at equal tension. This means that the resistance of the resting skeletal muscle is compared either with that of the isometric or with that of the isotonic contracted muscle. However, the difference in resistance between the isotonically contracted and resting muscle is much smaller than that between the isometrically contracted and resting muscle. The resistance of the isometrically contracted muscle when its length is intermediate between the equilibrium length and the resting length is at least 5 times greater than that of the comparable resting muscle (GASSER and HILL,[61] BUCHTHAL,[30] REICHEL[154]), whilst the isotonically contracted muscle seems never to attain double the value of the resting muscle (BUCHTHAL,[30], [31], [32] REICHEL[154]). It has only quite recently been shown that the active state of the contractile substance sets in about the middle of the latent period. Consequently, in the second half of the latent period, the resting and the active muscle have the same length and tension. Under these circumstances the resistance of a stimulated muscle to a quick stretch is about 50 per cent higher than that of a resting muscle (A. V. HILL[91]).

The fact that the contracted muscle shows an extreme variability in its resistance to stretch, according to the circumstances, arouses the suspicion that the resistance is not solely dependent on elasticity. This suspicion is confirmed by the following observations made by A. V. HILL,[83], [84], [85], [88], [89], [90], [93] ABBOTT, AUBERT and HILL,[4] ABBOTT

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and AUBERT.[2] The contracted muscle takes up and gives out heat when its length is changed (negative and positive heat of shortening). The amount of heat is independent of the tension developed and the work done. This suggests that the changes in length in the contracted state are not only accompanied by elastic changes but also by changes in metabolism.

All these changes of the mechanical state depend apparently on reactions between ATP and actomyosin; for even the resistance to stretch of fibre models extracted by glycerol-water increases to 5 times its initial value if these fibres, containing ATP, are brought isometrically from a state of rest (in the presence of salyrgan) to a state of contraction (by removing the poison on addition of cysteine, Table 3, group 2).

TABLE 3

Resistance to stretch of the fibre model in contraction, relaxation and rigor (20°C) (PORTZEHL[143])

1	2	3	4	5	6	7	8
Experimental group	*State**	*Temp.* (°C)	$100L/L_0$	*Resistance to stretch in* $g \times cm^{-2} \times L \times \Delta L^{-1}$		*Column 5 / Column 6*	*Number of experiments*
				with ATP	*without* ATP		
1	Contracted Rigor	~ 20	60–70	2200 —	— 5400	0·4†	6 5
2	Contracted Uncontracted	~ 20	68 67	1100 250	— —	— —	1 1

* Rigor: 10^{-3} M phosphate *p*H 7 + 10^{-3} M $MgCl_2$.
Contracted: the same mixture + 3×10^{-3} M ATP.
Uncontracted: the same mixture + 3×10^{-3} M ATP + 4×10^{-4} M salyrgan.

† The fact the model in rigor is less extensible than the contracted one was first reported by A. WEBER.[192] As it was not known at that time that the model, without ATP was in a state of rigor, the wrong conclusion had been drawn that the contracted model—contrary to the living muscle—was softer than the uncontracted one (H. H. WEBER,[201] H. H. WEBER and PORTZEHL[204]).

The resistance of the contracted fibre also only reaches about 40 per cent of the resistance of the rigid fibre. The latter is 10 times less extensible than the resting fibre, hence there is a four-fold increase of the apparent modulus of elasticity in the transition from rest to the contracted state.

The thread model, with its low content of protein, seems to resist stretch in rigor, contraction or rest about 10 times less effectively than either the skeletal muscle or the fibre model of a warm-blooded animal (PORTZEHL[142], [146]).

(*c*) *The elastic limit in the presence and the absence of ATP*

The rigid and the living muscles do not only differ in their elastic resistance to stretch but also in their elastic limit and tensile strength.

Living muscles at rest, as well as during contraction, can be reversibly stretched by 50 to 100 per cent of their length without tearing (A. SZENT GYÖRGYI[174]), whereas the rigid muscle tears even when it is gently stretched. Besides, it must be stretched very slowly if the stretch is to be reversible. This difference, too, depends on the presence or absence of ATP: fibre models without ATP are torn by a sudden stretch of 3 to 4 per cent (A. SZENT GYÖRGYI[174]). If the stretching is carried out very slowly the model fibre without ATP can be stretched by 30 to 40 per cent. But then the elastic limit is far exceeded: for

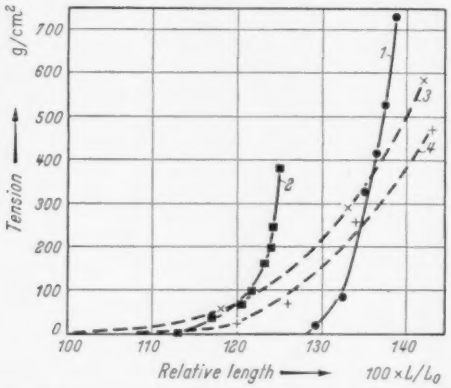

Fig. 2. Variation of tension with length on progressive release of the stretched model fibre (curves 1 and 2 according to A. WEBER[192] and the stretched rabbit psoas muscle (curves 3 and 4 according to SZENT GYÖRGYI[174]). For relative length see Fig. 1

on release the fibres shorten only incompletely (see Fig. 2). Moreover, this kind of stretching makes the fibres inhomogeneous to polarized light, showing that the structure of their contractile system has been damaged (A. WEBER[191], [192]).

(*d*) *The state of rest, contraction, rigor, and contracture.*

1. Owing to the plasticizing effect of ATP, the contractile system of the muscle containing ATP can be stretched by 50 to 100 per cent without being damaged. It is immaterial whether the system is at rest or in a shortened state.

2. The plasticizing activity of ATP reduces the resistance to stretch; in the resting state, to at least one tenth and in the contracted state to half the value found in rigor. This applies to measurements carried out in all cases where the system had the same relatively small initial length (for instance the equilibrium length).

3. When the ATP is exhausted or removed the contractile system becomes rigid. Its tensile strength, its elastic limit and its extensibility are small, but its resistance to stretch is great.

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The contractile system becomes rigid in the state of shortening and tension in which it happens to be at the moment when the content of ATP disappears. When the contractile system becomes rigid whilst it is in a state of contraction rigor is accompanied by contracture. This is often the case when the disappearance of ATP is due to a rapid breakdown by chemical agents or by chemical agents combined with a stimulation of the muscle (Lundsgaard effect). However, rigor is not accompanied by contracture if the concentration of ATP decreases very slowly. Thus rigor mortis appears without contracture at low temperatures or if, after death, the decomposition of ATP is slowed down by an abundant formation of lactic acid (BATE-SMITH and BENDALL,[15], [16] BENDALL[17]). On the other hand, the Lundsgaard effect shows a coupling of rigor with contracture because the formation of lactic acid is inhibited. Since carbohydrate now ceases to be a source of energy, the decomposition of ATP proceeds very quickly. The selective inhibition of the restitution of ATP depends on the fact that the restoring processes are much more sensitive to substances producing the Lundsgaard effect (fluoride, thioloprive substances, GODEAUX[66]) than is the ATPase (BAILEY and MARSH[11]).

It is therefore advisable to differentiate clearly between contracture and rigor, and the combination of both, even in those cases in which they normally occur together. Whereas the onset of rigor is necessarily coupled with the disappearance of ATP, the appearance of contracture is dependent on additional circumstances.

(*e*) *Analysis of the plasticizing effect of ATP and other polyorthophosphates*

A polyorthophosphate chain is evidently a prerequisite of the plasticizing effect; for ATP in its capacity as plasticizing agent can be replaced by pyrophosphate, triphosphate, ADP (PORTZEHL[146]), and apparently also by ITP. The latter produces a drastic contraction of the fibre model (LAJTHA,[108] SPICER and BOWEN[166]); but a high-grade shortening can only occur if the system has been in a plastic condition before. Further, all plasticizers, including ITP, reduce the cohesional forces between L-myosin and actin in actomyosin *solutions* in the same characteristic way. They bring about the dissociation of actomyosin (STRAUB,[170] DAINTY *et al.*,[40] SZENT GYÖRGYI and co-workers,[178] MOMMAERTS,[124] SNELLMAN and ERDÖS,[164] H. H. WEBER[199]). So far as a quantitative comparison has been made, ATP is superior to all other plasticizers (PORTZEHL[143], [146]).

What chemical groups of actomyosin are bound with ATP in the plasticizing reaction is not yet known. It is certain that *none* of the SH-groups of actomyosin are concerned which are indispensable for the *splitting* of ATP and which are blocked by salyrgan; for poisoning by means of salyrgan does not inhibit the plasticizing effect of ATP, and

an addition of salyrgan does not alter the rigidity of actomyosin (PORTZEHL[143], [146]).

III. Comparison of the states of contraction in the muscles and models

By means of extraction with glycerol-water mixtures contractile fibres have so far been obtained from the psoas muscle of the rabbit (see SZENT GYÖRGYI and co-workers,[176] H. H. WEBER and PORTZEHL,[205] further KOREY[101]), the m. sartorius, rectus, anconaeus, and cutaneus pectoris of the frog (PORTZEHL,[146] appendix BRECHT, BRECHT and EPPLE[29]), the muscle of *limulus* (A. SZENT GYÖRGYI,[176] SARKAR[160]) further from the posterior adductor and retractor pedis muscles of *Anodonta* and the longitudinal fibres of cow's rectum (G. and M. ULBRECHT[184]). Oriented threads have been prepared only from the actomyosin of the rabbit.

(*a*) *Tension and shortening*

Models of both types contract when treated with ATP, and both catalyze its hydrolysis. The maximal tension developed by the fibre model in this contraction is nearly the same (0·5 kg/cm² to 5 kg/cm²) as that developed by the living muscle from which the model was prepared (see Table 4). The maximal tension developed by the model

TABLE 4

Maximal tension and shortening of fibre models from cross striated and smooth muscles (H. H. WEBER and PORTZEHL[205])

Fibre models prepared from	*Maximal tension* (kg/cm²)	*Maximal shortening as %* (*of equilibrium length*)
skeletal muscle		
Rabbit	4*	~ 85*‖‡
Frog	1†	~ 70‡
smooth muscle		
Anodonta: adductor: yellow	5§	> 80§
white	2§	> 80§
Anodonta retractor pedis	—	> 70§
Cow rectum (longitudinal muscle)	0·6§	> 80§

* A. WEBER.[193] † BRECHT and EPPLE.[29] ‡ SZENT GYÖRGYI.[176]
§ G. and M. ULBRECHT.[184] ‖ VARGA.[188]

from skeletal muscle is reached at 18°C in a bath of a physiological concentration of ATP (5 to 8 × 10^{-3} M, Fig. 3*a*; see also A. WEBER[192]) and by the model from smooth muscles in a 2 to 3 × 10^{-3} M solution of ATP (G. and M. ULBRECHT,[184] Fig. 3*b*). The maximal tension reached by the skeletal muscle model (but not by the smooth muscle

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model) is limited by its tensile strength (Fig. 3*a*). The same applies to the thread model obtained from the actomyosin of the rabbit, the active maximal tension of which amounts to 200 to 300 g/cm² (Fig. 4).

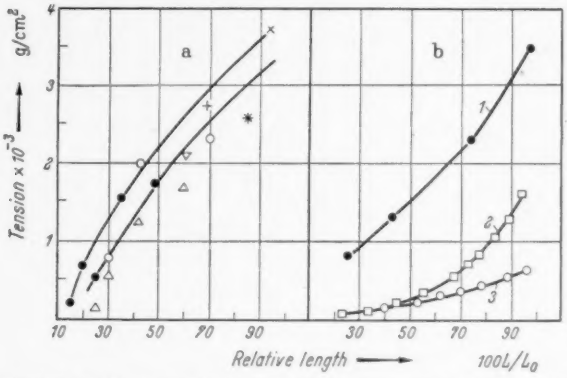

Fig. 3. Tension of fibre models developed in presence of ATP. (*a*) prepared from skeletal muscle of rabbit ×, +, *, torn fibres (A. WEBER[193]). For relative length see Fig. 1). (*b*) prepared from smooth muscle, curve 1 yellow *anodonta* adductor, curve 2 white *anodonta* adductor and curve 3 cow rectum (G. and M. ULBRECHT[184]). Relative length ≈ length *in situ*)

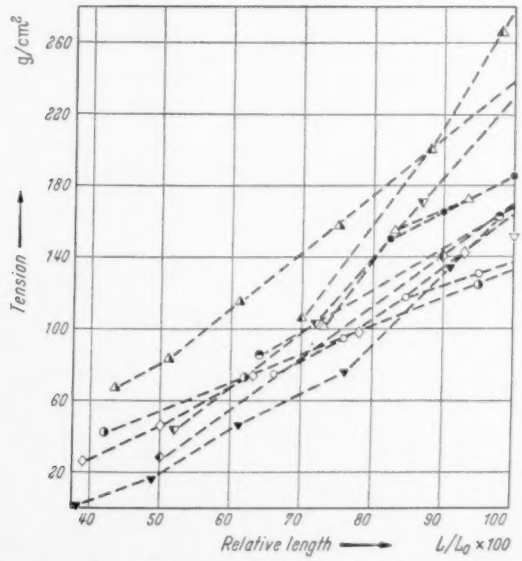

Fig. 4. Tension of actomyosin threads developed in presence of ATP (PORTZEHL[144]). (L_0 = equilibrium length)

The tension decreases proportionately with increasing shortening. The tension of the models from all sources becomes zero at 15 to 20 per cent of the equilibrium length, i.e. the maximal shortening of all fibre

models is about equal to the maximal shortening of smooth muscles (Table 4); whereas the maximal shortening of the thread model is only 60 to 70 per cent of the equilibrium length (Fig. 4).

If the actomyosin composing the thread is not directly isolated from the muscle extract, but is reconstituted from a pure monodisperse solution of L-myosin (PORTZEHL,[141] PORTZEHL *et al.*[147]) and a solution of actin (prepared according to STRAUB,[168], [169] A. SZENT GYÖRGYI[173]), mixed in the proportion of 3·8 mg L-myosin to 1 mg actin, the maximal

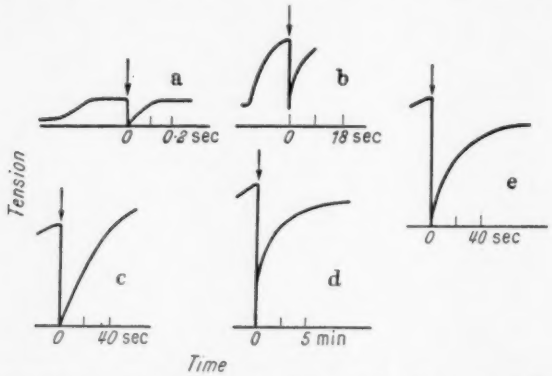

Fig. 5. Tension after quick release. (*a*) frog muscle (GASSER and HILL[61]) ↓ release from 100 to 90 per cent of relative length, (*b*) body wall muscle of holothuria (HILL[81]) ↓ release ~ 4 per cent, (*c*) fibre model prepared from rabbit psoas (A. WEBER[192]) ↓ release from 72 to 67 per cent, (*d*) fibre model prepared from cow rectum (G. and M. ULBRECHT[184]) ↓ release from 97 to 87 per cent, (*e*) thread prepared from a solution of rabbit actomyosin (PORTZEHL[144]) ↓ release from 94 to 91 per cent

tension seems to be much smaller, though the maximal shortening is the same (PORTZEHL[144]).

The tensions which have been discussed were always recorded with models which were allowed to shorten progressively. If, after shortening, the model is stretched again, a higher tension corresponds to the same length. The difference between the values obtained by the two procedures is greater the more rapidly the alterations in length are made (A. WEBER,[192] PORTZEHL[144]). The same applies to the intact muscle during contraction (LEVIN and WYMAN,[111] ABBOTT and AUBERT[3]).

(*b*) *The quick release phenomenon*

If a skeletal muscle in isometric tetanus is quickly released, so that it can shorten by 5 to 10 per cent of the standard length, the tension is completely abolished and then rises again (Fig. 5*a*) (GASSER and HILL[61]). Smooth muscles show the same phenomenon (Fig. 5*b*) (A. V. HILL[81]). All systems of models, i.e. fibre models prepared

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from cross-striated muscles (Fig. 5*c*) and from smooth muscles (Fig. 5*d*), as well as thread models (Fig. 5*e*), when under the influence of ATP, show the same effect if they are suddenly released from an isometric ATP contraction. But the tension of all the models returns more

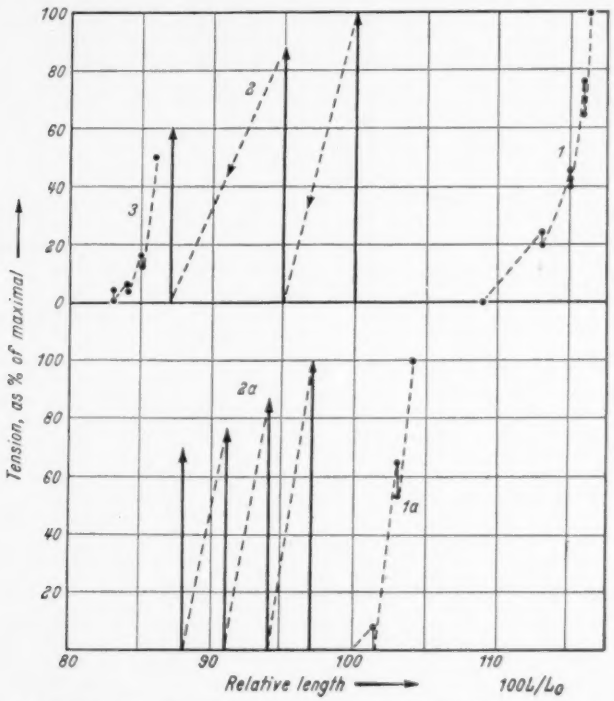

Fig. 6. Redeveolpment of tension in presence of ATP and elastic after-effect in the absence of ATP, following release. Curve 1: stretched fibre without ATP. Curve 2: unstretched fibre with ATP. Curve 3: unstretched fibre after washing out ATP (A. WEBER[192]). Curve 1*a*: stretched actomyosin thread without ATP. Curve 2*a*: the same, unstretched, with ATP (H. H. WEBER and PORTZEHL[204])

slowly to its former value than does the tension of living muscles (Fig. 5 and Table 11 in section C, III).

This phenomenon can only be found in muscular contraction, *in vivo* or in models, and cannot be imitated by any other elastic system. It cannot even be produced without ATP either with the mechanically stretched resting muscle or with the mechanically stretched model (Fig. 6). Here the tension falls after release to only a little below the new equilibrium value, which is finally reached by a small elastic after-effect, similar to W. KUHN and HARGITAY's models prepared from polyacrylic acid.[104] The quick release phenomenon is just as characteristic and specific a property of actomyosin systems during activity as is the contraction itself.

(c) *Temperature dependence of the contraction*

The tension of an uncontracted, slightly stretched actomyosin system (muscle as well as model) depends only slightly on temperature between 0 to 20°C. The tensions of living skeletal muscles and their models increase a little with temperature (curve 4 and 4*a* of Fig. 7 for muscle)

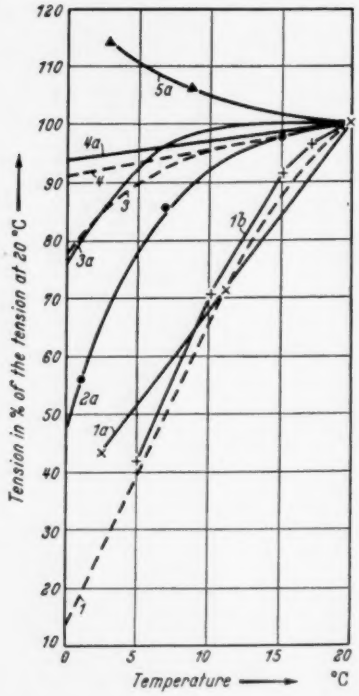

Fig. 7. Temperature dependence of tension. Curve 1: tetanized diaphragm (rat), (HAJDU[68]). Curve 1*a*: fibre model (rabbit), (A. WEBER[192]) and curve 1*b*: actomyosin thread (rabbit), (PORTZEHL[144]), both in 3×10^{-3} M ATP. Curve 2*a*: fibre model (smooth muscle, *Anodonta*), (G. and M. ULBRECHT[184]) in $2{\cdot}5 \times 10^{-3}$ M ATP. Curve 3: muscle in tetanus (frog), (A. V. HILL[92]). Curve 3*a*: muscle in thawing contraction (frog), (HAJDU[69]). Curve 4: resting muscle (frog), (JOSENHANS[98]). Curve 4*a*: fibre model without ATP (rabbit), (A. WEBER[192]). Curve 5*a*: fibre model without ATP (smooth muscle, cow rectum), (G. and M. ULBRECHT[184])

(see also WÖHLISCH and GRÜNING,[209] KNAPPEIS[100]). The tensions of living smooth muscles (G. ULBRECHT[183]) and their models decrease a little with a rise in temperature (Fig. 7, curve 5*a*).

If the skeletal muscle is stimulated with an appropriate frequency, i.e. high enough to anticipate decay of the active state, and if the development of the tetanic tension is thus kept as undisturbed as possible temperature dependence of the tension becomes considerably larger. The same is true of the models as soon as they are in a contracted state.

It is interesting that the tensions of models from smooth muscles also increase with a rise of temperature, although their tension in an uncontracted state falls with a rise of temperature.

The temperature dependence of tension during activity differs markedly in different muscles and their models. The tension developed

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both by the skeletal muscles and by the models from warm-blooded animals increases about 5 times between 0 and 20°C (curve 1, 1*a*, 1*b* of Fig. 7). For the model from the smooth muscle of *Anodonta* and from cow's rectum the tension increases 1·8 times between 0 and 20°C (curve 2 of Fig. 7) and in the vital and model contraction of the skeletal muscle from frog about 1·3 times (curve 3, 3*a* of Fig. 7). But in all tested cases the dependence on temperature both of the active model and the active muscle is quantitatively the same. The fact that this is also true of actomyosin threads (compare curve 1, 1*a*, and 1*b* of Fig. 7) denotes that differences in the temperature dependence between different species are due to a difference in the properties of the actomyosin itself rather than to morphological differences of the mechanical complexity of the muscles.

Just as the tension rises with temperature so does the degree of shortening of the models and muscles during contraction (VARGA,[186] HAJDU,[68] VARGA[187]).

According to SZENT GYÖRGYI and co-workers, above the optimum temperature both tension and degree of shortening decrease with increasing temperature, both in tetanus and during the contraction of models (A. SZENT GYÖRGYI,[176] HAJDU,[68], [69], [70] VARGA[188]). The optimal temperature for frogs is exceeded at 20°C in summer and in winter at 15°C (HAJDU[70]), for warm-blooded animals at about 30°C. Yet these data seem to require further investigations for three reasons: 1. The model denatures very rapidly at temperatures above 20°C (MATOLTSY[119]). 2. PORTZEHL[144] and H. H. WEBER[201] find that the degree of shortening of the actomyosin thread never decreases with a rise of temperature, whereas the tension falls only if it has previously been high. 3. A. V. HILL[92] did not observe a temperature optimum for the tension of the tetanized frog muscle up to a temperature of 28°C.

(*d*) *Birefringence and its change during the contraction of skeletal muscles and fibre models*

Not only are the mechanical properties of contracting muscles and their models the same or similar, but models and muscles resemble each other in molecular structure both in the resting state and during contraction. This can be deduced from birefringence studies of the muscles of warm-blooded animals and their fibre models.

The birefringence of muscle decreases only very slightly in isometric contraction (v. MURALT[132]), but very considerably when shortening takes place (v. EBNER[48]). The change observed is determined overwhelmingly by the change in length and only insignificantly by the transition from the inactive to the active state. The same applies to the fibre model (H. H. WEBER[202]).

The intact muscle of warm-blooded animals and its model show, when uncontracted, the same intrinsic birefringence and the same form birefringence, as well as total birefringence (Table 5). In isotonic contraction, too, the total birefringence of the model is quantitatively

the same as that of the muscle. Therefore the assumption seems to be justified that in contraction also the intrinsic and form birefringence of both muscle and model are identical. Only in models (even when contracted) can the intrinsic and form birefringence be measured separately. For only during the contraction of the model can the system be fixed in every state of shortening and tension by washing out the ATP. During the isotonic contraction of the fibre model soaked in water, intrinsic and form birefringence decrease nearly proportionally (Table 5).

TABLE 5

Birefringence of skeletal muscle and fibre model (H. H. WEBER and PORTZEHL[205])

	Contracted		*Uncontracted*	
	model	*muscle*	*model*	*muscle*
Intrinsic birefringence $\times 10^3$ . .	0·68*	—	1·1*	0·85‡
Form birefringence $\times 10^3$ . . .	0·74*	—	1·6*	1·65‡
Total birefringence $\times 10^3$ . .	1·4*	1·4†	2·7*	2·50‡

* STRÖBEL.[171] † v. EBNER.[48] ‡ E. FISCHER.[55]

Analysis of the change in birefringence only results in a correct analysis of the change in the molecular structure of the contractile system when, in addition to the changes in birefringence in water, those in media of a different refractive index are taken into account. It then becomes evident that during contraction intrinsic birefringence decreases to half its value in all media (Table 6, last column); whereas form birefringence in all organic media ($n_2 = 1{\cdot}45$ to $1{\cdot}68$) remains unchanged and diminishes only in water and ethanol (compare row 1 with row 2 of Table 6).

Now for some time it has been ascertained by comparing the dependence of birefringence on swelling on the one hand and the x-ray diagram on the other that bodies composed of protein rods (*Stäbchenmischkörper*) take up water also into the single rod. Intramicellar swelling occurs only if the medium is water or ethanol (H. H. WEBER,[196] compare also NOLL and H. H. WEBER[135]). It reduces the refractive index of the protein rods, thereby diminishing also form birefringence, even if the shape and arrangement of the rods do not change at all (H. H. WEBER[196]).

The decrease in birefringence in an aqueous medium only denotes that the rods take up more water on shortening. However, the fact that the form birefringence remains constant in organic media of a higher

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refractive index indicates that the protein rods remain oriented parallel to the axis of the fibre during contraction; for these media penetrate only into the interstices between the rods. Nor does the orientation of the rods alter if the actomyosin systems are passively stretched (H. H. WEBER,[196], [197] ASTBURY and DICKINSON[8], [9], [10]).

TABLE 6

Changes in intrinsic and form birefringence during contraction of the fibre model. (All values are calculated for the protein concentration of 15 per cent). (STRÖBEL[171])

	Medium	*Form birefringence* $\times 10^3$							*Intrinsic birefringence* $\times 10^3$
		Water	*Ethanol*	*Ethanol + Aniline*		*Aniline + Bromonaphthalene*	*Bromonaphthalene*	*Bromonaphthalene + Methyleneiodide*	
	Index $= n_2$.	1·33	1·36	1·45	1·56	1·61	1·65	1·68	
1	Uncontracted . (3 fibres)	1·63	1·02	0·28	0·00	0·44	0·98	1·20	1·13
2	Contracted . (4 fibres)	0·74	0·54	0·23	0·05	0·30	1·20	1·37	0·68

From the viewpoint of molecular morphology the results of birefringence studies indicate that active contraction and passive stretching are opposite processes, i.e. the peptide chains, oriented parallel to the axis of the fibre in the single protein rod, pass during contraction from an intermediate to a strongly folded state, while they are straightened out on stretching. This change in molecular folding is the cause of the change in intrinsic birefringence. Neither shortening nor lengthening alter form birefringence in organic media, because the rods as a whole retain their axially parallel orientation, and their length, even in a shortened state, still remains large enough to satisfy the supposition on which the Wiener equation is based.

This conclusion can be strengthened to a certain extent by study of the electron microscopic pictures. The smallest protein filaments which are still resolvable in the electron microscope are always straightened out in the stretched, normal, and contracted states and run parallel throughout the whole fibril and as a whole become only longer or shorter (DRAPER and HODGE[43], [44]). Birefringence studies, however,

extend the last observation to those protein particles which can only be investigated by this method, and not by means of the electron microscope. For the high birefringence of the A-bands is apparently not produced by the protein filaments which run through the A- as well as the I-bands, but by other protein rods which are only to be found in the A-bands and which cannot be resolved in an electron microscope.

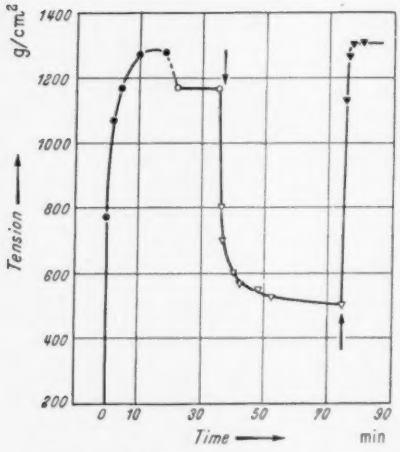

Fig. 8. Relaxation of fibre model by adding pyrophosphate (H. H. WEBER[202], PORTZEHL[146]). • – • – • – ATP-contraction ($2 \cdot 10^{-3}$ M ATP). – – – – washing out, ○ – ○ – ○ – without ATP. ↓ = 0·025 M-pyrophosphate, ↑ = $3 \cdot 10^{-3}$ M ATP anew

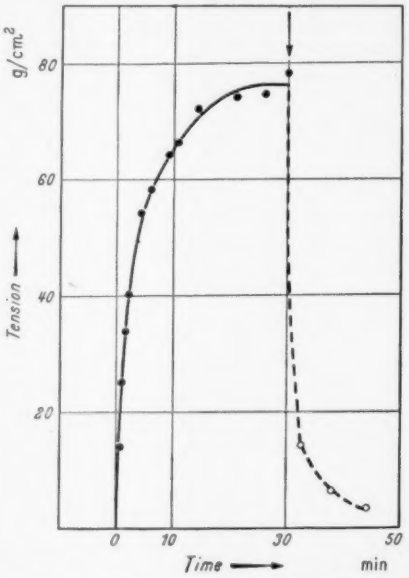

Fig. 9. Relaxation of thread model by adding sodium triphosphate (PORTZEHL[146]). ↓ ATP is washed out by 0·04 M $Na_5P_3O_{10}$. For the rest compare Fig. 8

C. The Phase of Relaxation and the Energetics of the Working Cycle

I. Relaxation of the models in presence of plasticizers

(a) Relaxation when other plasticizers replace ATP

When ATP is washed out of a contractile model, the tension and length remain largely unchanged (as in the thread model), or sometimes entirely unchanged (as in the fibre model), (compare Fig. 8) (A. WEBER,[192] PORTZEHL,[143, 146] H. H. WEBER,[202] SZENT GYÖRGYI[176]). This fact has given rise to various speculations as to what special conditions are necessary to cause the relaxation of the living muscle

(SZENT GYÖRGYI[173], [176]). Actually this incapacity to relax is due only to the rigidity of the muscles and models, which occurs when all plasticizers are removed (see section B, II).

The rigidity of the contracted thread or fibre model is destroyed by the addition of plasticizers which do not simultaneously cause contraction. Substances of this kind are pyrophosphate, triphosphate or ADP. They cause both thread and fibre models, prepared from cross-striated or smooth muscle, to relax (Fig. 8 and Fig. 9) (see also H. H. WEBER,[202] BOZLER,[27] G. and M. ULBRECHT[184]). This relaxation is occasionally not as complete as that initiated by adding ATP, because the other

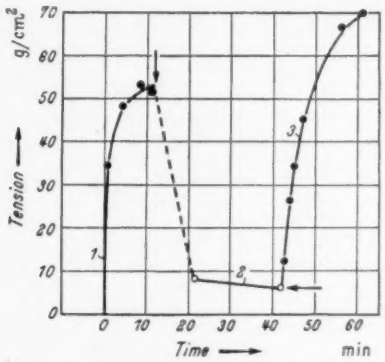

Fig. 10. Contraction and relaxation in presence of a constant concentration of pyrophosphate ($1{\cdot}5 \cdot 10^{-2}$ M) (H. H. WEBER[202], PORTZEHL[146]). First contraction with $1{\cdot}6 \cdot 10^{-3}$M ATP. ↓ ATP removed, ← ATP anew ($4 \cdot 10^{-3}$ M). Thread model

polyphosphates are less efficient plasticizing agents. These substances can easily be shown to act only as plasticizers, and not by providing energy which might be required for relaxation. If the model is kept in a solution of pyrophosphate before the addition of ATP and if the concentration of pyrophosphate is held constant throughout the course of the experiment, contraction nevertheless occurs on the addition of ATP and relaxation after the removal of ATP. Thus relaxation takes place, although the moment when the pyrophosphate might have become bound to the actomyosin with a possible release of energy has long been over (Fig. 10). Even very unspecific plasticizing effects can be shown to suffice for a reversible relaxation, e.g. HAYASHI'S[78] contracted actomyosin thread, which is rigid after removal of ATP, can be made to relax reversibly by means of an increase of ionic strength to 0·3. Similarly BOZLER'S[27] fibre model relaxes reversibly in urea.

Consequently relaxation is merely the cessation of contraction—without terminating in rigidity. Relaxation is not an active process, but the thermodynamically spontaneous phase of the working cycle.

This has already been contended by HILL.[87] He based his conclusion on the following observations: 1. The living muscle produces primary heat only during contraction (A. V. HILL,[83], [84], [85], [88], [89], [90], [93] ABBOTT,[1] FENN[54]). 2. The shortened muscle does not lengthen after relaxation, unless stretched. The second observation, however, applies to the muscle as a whole (W. KÜHNE,[102] A. V. HILL[87]), not to the isolated living fibre in Ringer's fluid (RAMSEY and STREET[151], [152]). HILL, trying to explain this discrepancy, argues that the fibres inside a whole muscle, surrounded by their own lymph, are under better physiological conditions than the isolated fibres in Ringer's fluid, which for instance has no colloid-osmotic pressure (HILL,[87] but see BUCHTHAL and KAISER,[35] page 191).

However this may be, HILL's conception that relaxation is not an active process is confirmed by the observation of the isolated working cycle obtained by studying simplified muscle models.

But if relaxation is the thermodynamically spontaneous part of the working cycle, contraction can occur only when free energy is supplied to the contractile mechanism. This answers the question as to whether the energy from metabolism is transmitted to the contractile protein during *contraction* and immediately used (HILL,[87], [88], [92] H. H. WEBER,[198], [203] A. WEBER and H. H. WEBER,[194] DUBUISSON[46]) or whether it enters into the contractile protein during *relaxation* and is stored up for a future contraction (BETHE,[21] WÖHLISCH,[207] W. KUHN and HARGITAY,[104] M. PRYOR,[149] GUTH,[67] A. WEBER and H. H. WEBER[193]).

(*b*) *Relaxation when ATP splitting is poisoned. The thermodynamics of* SZENT GYÖRGYI, WÖHLISCH *and* PAULING

ATP has a dual role. It induces the actomyosin thread and the fibre model to contract, and simultaneously it is the most effective plasticizing agent of all polyorthophosphates. This dual role serves a purpose: the contractile system must be in a plastic condition in both cases—whether contracting or relaxing. The question arises whether it is possible to separate the effect of ATP as a contracting agent from its effect as a plasticizer, in such a way that it can also serve as a relaxing agent.

If the fibre model which has become rigid in contraction by the removal of ATP is poisoned by salyrgan, no alteration takes place. But by adding ATP again the model at once relaxes (Fig. 11). The relaxation is not complete, however, due to the conditions of the experiment which allow time for the salyrgan to diminish the plasticizing effect of ATP (PORTZEHL[143], [146]). This time is shortened if the model is poisoned by salyrgan at the height of contraction without ATP having first been washed out. Consequently, under these conditions, both the fibre

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model (Fig. 12*a*) and the thread model (Fig. 12*b*) relax almost completely.

However, the relaxing effect of ATP always turns immediately into a contracting effect of ATP as soon as the effect of salyrgan is inhibited by an addition of cysteine (see Figs. 11, 12*a* and 12*b*). The effect of salyrgan is to poison the *splitting of ATP* by actomyosin (KUSCHINSKY and

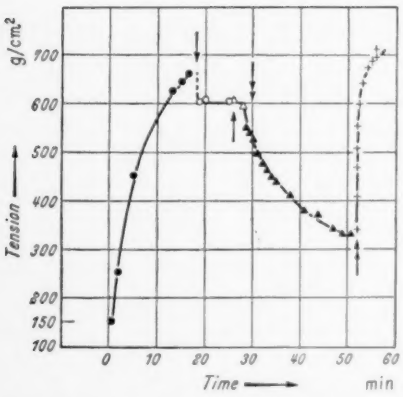

Fig. 11. Relaxation produced by ATP after poisoning with salyrgan (PORTZEHL[146]). First contraction with $1{\cdot}7 \cdot 10^{-3}$ M ATP, ↓ ATP is washed out, ↑ addition of salyrgan $6{\cdot}6 \cdot 10^{-4}$ M, ↓ addition of $1{\cdot}7 \cdot 10^{-3}$ M ATP again, ↑ addition of $6{\cdot}7 \cdot 10^{-2}$ M Cysteine. Fibre model

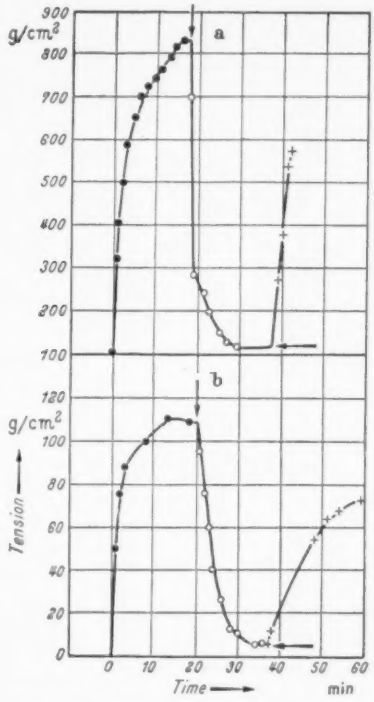

Fig. 12. Relaxation produced by salyrgan. (*a*) fibre model, (*b*) thread model (PORTZEHL[146]) ↓ $1 \cdot 10^{-4}$ M salyrgan, ← $2 \cdot 10^{-2}$ M cystein

TURBA[105]).* Accordingly ATP, in the presence of Mg, is a contracting agent as long as it is split, but a plasticizer when not split.

The splitting of ATP also diminishes strongly if the ATP concentration is greater than optimal (HASSELBACH[73] and HEINZ and HOLTON[79]). Therefore ATP becomes also a relaxing agent if more than the optimal

* The ATPase poison, benzaldehyde, (KUSCHINSKY and TURBA[105]) also causes a complete relaxation of the contracted model (PORTZEHL[143], [146], H. H. WEBER[202]). Since the relaxation is irreversible and benzaldehyde is itself an excellent plasticizer, this result can theoretically be explained in different ways.

concentration is added to a fibre model (BOZLER,[27] A. WEBER and H. H. WEBER[194]) (Fig. 13).

Thus we see that the contraction of the models is closely coupled with a steady state of ATP splitting; on the other hand SZENT GYÖRGYI and co-workers[175], [176], [177] assume in their thermodynamical speculations that the contracted state of the muscle is a new state of equilibrium and that this equilibrium depends on temperature in a way which is thermodynamically reversible. SZENT GYÖRGYI[177] holds this assumption to be correct, because in a contracted fibre model, the same tension is always coordinated to a given temperature, regardless of whether the latter has been reached from above or below. In fact,

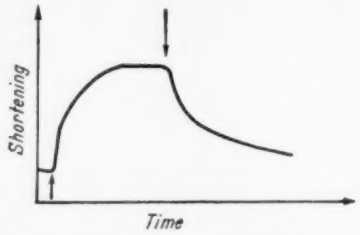

Fig. 13. Relaxation produced by over-optimal ATP concentration. Fibre model (bundle of fibres, BOZLER[27]). Isotonic contraction loaded with 1 gm, ↑ 0·2 per cent ATP, ↓ 1·5 per cent ATP

the tension is exactly coordinated to the temperature (SZENT GYÖRGYI,[174,] [177] A. WEBER and H. H. WEBER,[193], [194] PORTZEHL and H. H. WEBER,[148] VARGA[187], PORTZEHL[144]). This phenomenon, however, seems not to be based on a state of thermodynamical equilibrium but on the fact that a definite rate of ATP splitting corresponds with each temperature (see section C, II). Consequently, SZENT GYÖRGYI'S reasoning is not conclusive.

E. WEBER'S[206] (1846) old conception of the muscle being in a new elastic state during contraction has twice been developed into a more precise form in modern colloid chemistry: 1. WÖHLISCH'S[207] thermokinetic theory of muscular contraction. (See also W. KUHN and HARGITAY,[104] M. PRYOR,[145] GUTH[67]). 2. PAULING and COREY'S theory.[138] According to the latter theory the shortened structure of actomyosin has less chemical energy than the relaxed one.

These otherwise opposed theories agree fundamentally in the statement that contraction is the thermodynamically voluntary part of the working cycle and relaxation the thermodynamically involuntary one (i.e. when free energy is supplied). This would be incompatible with the above-mentioned facts. The thermokinetic theory, however, has been developed by KUHN and HARGITAY[104] as well as by MORALES and co-workers (see below) in such a way as to make it possible to bring their

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theory, by further assumptions, into line with the facts hitherto propounded.

II. Splitting of ATP and the working cycle

The fact that relaxation occurs spontaneously (see section C, I*a*) proves that the transfer of energy to the contractile system takes place during the phase of contraction and not during relaxation. The changes of *p*H, too, in the contracting living muscle make it extremely likely that ATP is split during the phase of contraction (DUBUISSON[45], [46]). Further, if living frog muscles are fixed by liquid air during contraction, their ATP content is smaller (LUNDSGAARD,[116] MOMMAERTS and RUPP[126]) but their content of ADP and AMP is larger (MOMMAERTS and RUPP[126]) than the content of these substances in the resting control muscle. Study of the isolated working cycle confirms conclusively that ATP is the only effective energy-provider. However, in order to become split ATP must previously be bound. The question therefore arises: is it the binding of ATP to the actomyosin or its catalytic splitting which supplies the energy for contraction (H. H. WEBER[202])?

In order to answer this question the following observations have to be taken into account:

1. All polyorthophosphates can be bound by actomyosin. This is shown by their plasticizing effect (see sections B II and C I*a*) as well as by their effect on the dissociation of dissolved actomyosin (MOMMAERTS,[125] SNELLMAN and ERDÖS,[164] H. H. WEBER,[199] KUSCHINSKY and TURBA,[106] TURBA *et al.*[182]). In spite of this, only ATP and ITP act as contracting agents, as they are the only two polyorthophosphates* which can be *split* by actomyosin (see PORTZEHL,[143], [146] and section B, II).

2. ATP can be bound by actomyosin in the presence as well as in the absence of salyrgan (sections B, II*e* and C, I*b*), for in either case ATP acts as a plasticizer. But as a contracting agent ATP causes contraction only when it is split. As soon as the hydrolysis of ATP is poisoned by salyrgan ATP acts as a relaxing agent (section C, I*b*).

The first observation and more especially the second show that a correlation exists only between the breakdown of ATP and contraction, but not between the binding of ATP and contraction. This is a strong argument in favour of considering splitting of ATP as the source of energy in contraction.

Even so, the following possibility cannot be ruled out entirely. ATP might be bound alternately by two different groups of actomyosin, for instance, it might at one time be bound so as to cause plasticity (bound in "plasticizing position"), or it might be bound so as to cause contraction (bound in "contracting position"), in which latter case it is

* The splitting rate of triphosphate observed by TARVER and MORALES[181] is more than twenty times smaller.

simultaneously split. The energy for contraction might then be released when ATP moves from the "plasticizing position" to the "contracting position". But this implies the unproved supposition that this transition sets free an amount of energy which is not much smaller than the energy released by ATP splitting (compare the efficiency of the ATP splitting, section C, III). The latter would then serve only to release the nucleotide from the binding in the "contracting position" in this way ensuring an unlimited supply of energy. Should there be a possibility of preventing the breakdown of ATP even when it is bound in the "contracting position", the result would be an ideal case of tone (*Sperrtonus*); in other words, the muscle would remain contracted without any liberation of energy.

Speculating in this way, ATP breakdown would no longer be the cause of contraction but would become only a necessarily attendant feature. The real cause of contraction would be the binding of ATP in its "contracting position". Such a theory is proposed by MORALES and co-workers in their modification of the thermokinetic theory (MORALES and BOTTS,[129], [130] T. HILL and MORALES[96]). This conception has to contend with the fact that so many factors influence alike both splitting and contraction; it is not very likely that each of these factors influences the splitting of ATP either by increasing or by inhibiting the transition from the "plasticizing" to the "contracting position". It is almost certain that some alter nothing but the velocity of breakdown of the enzyme-ATP-complex.

The following factors are known:

1. All substances which—like salyrgan—block or oxidize the SH-groups of actomyosin prevent ATP splitting and contraction (Table 7). It has been shown that the denaturation which occurs in those cases is the result of a loss of SH-groups—at least where this has been examined (Table 7, BAILEY and PERRY[12]). It may be possible that the SH-groups serve to bind the ATP to the actomyosin. In this case both splitting and contraction would be inhibited, since ATP could no longer be bound in a "splitting position" (= contracting position).*

2. *Influence of temperature.* If the temperature is gradually lowered from room temperature to 0° both the rate of splitting ($Q_{10} \sim$ to 2) and the intensity of contraction decrease, although it is most improbable that the amount of bound ATP becomes smaller with the fall in

* In this connection one is inclined to think of an esterification (BINKLEY[22]); but, if so, addition of ATP to actomyosin would produce a *p*H change. Such a *p*H change, however, was not noticed by FABRY-HAMOIR,[52] although the standard error of the technically excellent measurements did not exceed 0·003 *p*H units. On the other hand it is not known how much ATP may be bound by actomyosin. That all SH-groups do not participate in the binding of ATP is certain (SINGER and BARRON,[162] KUSCHINSKY and TURBA[105], [107]). According to KUSCHINSKY and TURBA those SH-groups which can be oxidized by ferricyanide to SS-groups are not necessary.

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TABLE 7

SH-binding and oxidizing effects on contraction and ATPase activity

Inhibition by	*Molarity of reagent*	*System employed*	*ATPase inhibition* (%)	*Contraction*		*Reversibility*
				Character of reaction	*Inhibition* (%)	
H_2O_2	4×10^{-3}	L-myosin†‡	90–100	—	—	None
	3×10^{-1}	Glycerol fibre*	—	Contraction*	+	None
	2×10^{-1}	Actomyosin thread§	—	Shrinkage§	100	None
O-iodosobenzoate	1×10^{-3}	L-myosin†	90	—	—	With 10^{-2} M cysteine
	1×10^{-3}	Glycerol fibre*	90	Contraction*	100	+
Iodoacetamide	4×10^{-2}	L-myosin†	9	—	—	By dialysis
	1×10^{-1}	Actomyosin thread§	—	Shrinkage§	100	None
Salyrgan	2×10^{-4}	Actomyosin‖	100	Superprecipitation‖¶	100	With 10^{-2} M cysteine
		Actomyosin thread**	—	Tension**	100	+
Cu-glycinate	5×10^{-4}	Actomyosin††	Strong	Superprecipitation††	Strong	+
Ageing (20 days at *p*H 7·4)	—	Glycerol fibre*	80	Contraction*	100	None
Precipitation at *p*H 5·2—						
15 min.	—	Actomyosin‡‡	~ 23	Superprecipitation‡‡	100	—
330 min.			~ 75			
Dialysis against H_2O at *p*H 6	—	Myosin§§	Strong	Shrinkage§§	0	None

* Korey.[101]
† Bailey and Perry.[12]
‡ Ziff.[210]
§ Godeaux.[66]
‖ Kuschinsky and Turba.[108]
¶ Kuschinsky and Turba.[106] [107]
** Portzehl.[146]
†† Turba *et al.*[182]
‡‡ Spicer.[165]
§§ Buchthal *et al.*[33]

temperature. This suggests that only the splitting of ATP is the immediate energy-source of ATP contraction.

In order to compare quantitatively the effect of temperature on the splitting of ATP with its effect on contraction it is necessary to measure the contraction in terms of power (i.e. as work per unit of time) because the rate of splitting gives the energy per unit of time. Suitable data are available only for the fibre model of the smooth adductor muscle from *Anodonta*. In this preparation both power and rate of splitting rise about 7 times when the temperature is raised from 0° to 20° (G. and M. ULBRECHT[185]). The temperature dependence of the mechanical and chemical effects agree quantitatively with each other (section C III).

3. *"Over-optimal" ATP concentration.* If the ATP concentration exceeds the optimal value the rate of breakdown falls again. It would contradict the law of mass-action to assume this decrease of breakdown to be dependent on the amount of ATP bound in the "splitting position". Nevertheless both the tension development by the fibre model and the analogous superprecipitation of the actomyosin gel diminish, too, with the decrease in splitting (Table 8). The limiting ATP concentration, at which this parallel decrease of both splitting and tension begins, varies greatly according to the experimental conditions: Temperature, ionic strength, and the thickness of the fibres or particles (Table 8) influence the results. For it depends on the thickness, that is on the length of the diffusion path, how much lower the ATP concentration is inside the model than in the bath. However, it must be stressed that under comparable experimental conditions, the decrease of contraction always begins at the same concentration of ATP as that at which the rate of breakdown of ATP begins to decrease.

TABLE 8

Limiting ATP-concentration beyond which decrease of contraction and ATP-breakdown begins (H. H. WEBER and PORTZEHL[205]).

Preparation	*Ionic strength* (μ)	Mg^{++} *molar*	Temp. (°C)	*Limiting* ATP-*concentration*		*Manifestation of contraction*
				Splitting molar	*Contraction molar*	
Fibre model	0·04	10^{-3}	20	$10^{-2\cdot0}$*	$10^{-1\cdot7}$†	Tension
	0·04	10^{-3}	2	$10^{-2\cdot3}$*	$10^{-2\cdot3}$†	
Actomyosin particles	0·09	0	5	$10^{-2\cdot5}$‡	$10^{-2\cdot4}$‡	Superprecipitation
	0·09	0	20	$10^{-2\cdot2}$‡	$10^{-2\cdot2}$‡	
	0·09	0	30	$10^{-2\cdot0}$‡	$10^{-2\cdot0}$‡	

* HEINZ and HOLTON.[79] † A. WEBER and H. H. WEBER.[194] ‡ BIRO and A. G. SZENT GYÖRGYI.[33]

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4. *The Marsh-Bendall-factor.* A "factor" can be extracted from the muscle which prevents not only the superprecipitation of actomyosin particles which are under the influence of physiological ATP concentration (MARSH[117], [118]) but also the development of tension by the fibre models (BENDALL[18] and HASSELBACH[75]). The same "factor" inhibits the splitting of ATP, and it does so to the same extent as it affects the development of tension (Fig. 14). When added at the height of contraction, the "factor" causes complete relaxation (HASSELBACH[75]).

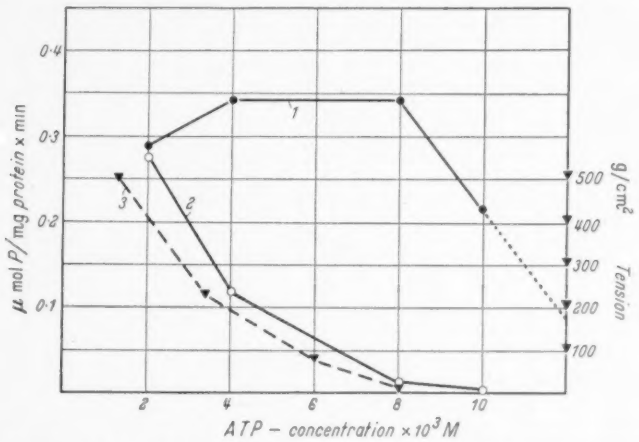

Fig. 14. The influence of M-B-factor on ATP splitting and contraction (HASSELBACH[75]). 1. Splitting without M-B-factor. 2. Splitting with M-B-factor. (1 and 2 refers to the left ordinate). 3. Tension with M-B-factor, refers to the right ordinate. Temperature 20°C, *p*H ~ 7, ionic strength ~ 0·11 μ. Curves 1 and 2 glycerol extracted fibres treated with blendor, curve 3 fibre model

The M-B-factor seems to act by preventing the breakdown of bound ATP, not by displacing bound ATP. For the inhibiting effect of this "factor" consists only in a shift of the optimal ATP concentration (see Fig. 14). The latter is shifted downwards so considerably that even the normal physiological ATP concentration of 7×10^{-3} M is so far above the optimum that both breakdown and contraction are totally blocked (see Fig. 14).

5. Finally, contraction and ATP breakdown are strongly influenced by the presence of ions, being sometimes activated and sometimes inhibited. However, with the exception of calcium, the influence on splitting and contraction are always parallel, (see Table 9).* A rise of

* For a time it seemed as if magnesium ions promoted contraction but inhibited ATP breakdown, and as if the optimum of *p*H's for splitting and contraction were different. Of late, however, it has been found that the *p*H optimum is the same (KIELLEY and MEYERHOF,[99] SARKAR *et al.*,[161] PERRY[139]) and that Mg even activates splitting (SARKAR *et al.*,[161] PERRY,[139] HASSELBACH[73]) provided that care is taken that the actomyosin gel remains completely undissolved also in presence of ATP (HASSELBACH[73]).

TABLE 9

Dependence of ATP-breakdown and contraction on the concentration of inorganic ions (temperature 20–25°C) (H. H. WEBER and PORTZEHL[205])

Ionic concentration employed	*Breakdown of ATP*	*Contraction produced by ATP*	*Character of reaction*	*Actomyosin system employed*
*p*H optimum in 0·1 M KCl + 10^{-3} M $MgCl_2$	~ 7*†	~ 7‡	Tension	Fibre model*† Thread model‡
0·01 → 0·2 M KCl (+ 10^{-3} M $MgCl_2$)	Maximal = 100%*	Maximal = 100%*§††	Tension*§	Fibre model*§
0·2 → 0·4 M KCl (+ 10^{-3} M $MgCl_2$)	Decrease from 100 to 15%*		Tension*	Fibre model*
0 → 0·1 M KCl without addition of $MgCl_2$	Increase to 100%‖		Shrinkage‖	Actomyosin thread unoriented‖
≦ 10^{-2} M $MgCl_2$	Activation*†¶	Activation‡§‖	Shrinkage‖ Tension‡§	Actomyosin thread unoriented‖ Thread model‡ Fibre model§
≦ 10^{-2} M $CaCl_2$	Activation†‖¶**	Inhibition‖	Shrinkage‖	Actomyosin thread unoriented‖

* SARKAR, SZENT GYÖRGYI and VARGA.[161] † PERRY.[138] ‡ PORTZEHL.[142] § A. WEBER.[191]
‖ BOWEN.[26] ¶ HASSELBACH.[78] ** BANGA and SZENT GYÖRGYI.[13]
†† SARKAR *et al.*[161] indicate, however, that the tension in solutions of concentrations between 0·01 and 0·1 M KCl is below the maximal value. This is contrary to the observations of A. WEBER[191] with fibre models and PORTZEHL[142] with thread models.

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Ca concentration increases the ATP splitting very strongly if no Mg is present (SPICER and BOWEN[166]) and does not promote contraction at all (but compare section C, V). The fact that the splitting energy can be increased by Ca over and above the amount necessary for contraction does not, of course, disprove that the splitting is the source of energy. It proves, however, that special conditions have to be observed, if the available energy of the ATP breakdown is to be actually used for contraction.

The energy of splitting released by denaturated fibres probably exceeds likewise the requirement of contraction, since the ATP splitting is kept considerably longer intact than contraction (GERGELY,[63] LAJTHA,[109] PERRY[139]). There as in the Ca activity the problem of the efficiency of ATP breakdown appears.

The binding of ATP in a contracting position could soon be shown to be the immediate source of energy if one could succeed in blocking the ATP splitting completely without preventing contraction. So far this has not been achieved.

Yet, one observation has been recorded according to which actomyosin threads are still capable of shrinking as much as normal threads even if the ATPase activity of the latter has been largely destroyed (see Table 7) (BUCHTHAL *et al.*[33]) by dialysis at *p*H 6 (according to the procedure of SINGHER and MEISTER[163]).*

Since, however, this shrinkage did not take place against a tension, its energy requirement is small and difficult to estimate. Considerable changes in the energy available from decomposition could lead only to small changes in shrinkage. Besides SZENT GYÖRGYI reports the opposite, i.e. that the contractility disappears when the ATPase activity is lowered to ≤ 50 per cent of its normal value by acidification (SZENT GYÖRGYI,[173] page 121).

To summarize: none of the reported facts suggests that there is a transfer of the contracting energy from ATP to actomyosin by means of ATP binding, whereas a great number of the above-mentioned facts are in favour of the suggestion that the energy set free by splitting is the direct source of contraction. Still, the possibility that the contracting energy is released by the binding of ATP cannot be as definitely excluded as, for instance, the possibility of a transmission of energy in the phase of relaxation (see section C Ib).

III. Speed of shortening and efficiency of the isolated working cycle

It is well established that the energy required for the isolated working cycle ultimately results from the breakdown of ATP. It would therefore be useful to arrive at an estimation of the efficiency of this energy. It is unimportant as regards the efficiency whether splitting energy or

* MOMMAERTS[125] found it impossible to destroy the ATPase activity of actomyosin *completely* by acidification—even after precipitating 3 times at *p*H 5·2.

binding energy is immediately transferred to the contractile substance. Since a continuous binding of ATP is only made possible by a continuous splitting of ATP, the release of binding energy is determined by the release of splitting energy. On both views the amount of energy released will be the same, only the mechanism will be different. In the one case the molecular change of structure is associated with the formation of an actomyosin ATP complex, in the other with the decomposition of the same complex.

TABLE 10

Speed of shortening, power and efficiency of the model (at 20°) and *in vivo* (at 0°) contraction

Fibre model prepared from	*Tension* (kg/cm^2)	*Speed* ($100\Delta L/L$) sec^{-1}	*Shortening from to*	*Power* ($\mu cal\ sec^{-1}\ cm^{-3}$ model)	*Efficiency* (%)
Rabbit* . . .	0·50	~ 5·0	80–70%	~ 600	~ 6·0
	0·50	~ 10·0	80–70%	~ 1200	~ 13·0
	1·00	~ 1·5	80–70%	~ 350	~ 3·5
Anodonta adductor .	—	~ 10·0†	**100–50%	—	—
Cow rectum (longitudinal muscle)	—	2·5†	100–75%	—	—
Living muscle (frog) .	~ 0·00	100‡ → 200§	~ 100–80%	—	(anaerobic)
	~ 0·02	185§	~ 100–90%		20–30‖

* FALK.[53] † G. and M. ULBRECHT.[154] ‡ ABBOTT and RITCHIE.[6] ‖ MEYERHOF.[120] § HILL.[94]
** The length of the muscle in relaxation produced by narcotics is here taken as the standard length. It is 110 to 115 per cent of the length of the muscle in closed shells.

In order to compare the rate of splitting with the power of the isolated working cycle, besides tension and degree of shortening, the speed of shortening under a given load must be known. The measurement of this is difficult, as it has to be carried out with microscopic preparations of fibres ($2r \leq 30\mu$) if the whole cross-section is to be soaked with ATP (see section B, Ib). It is especially difficult as the outer layers of the models begin to contract as soon as the ATP reaches them, while the centre of the model is not reached by the ATP until 5 to 10 seconds later. This means that the contraction at first occurs against the resistance of the rigid centre, therefore with reduced speed. This fault is diminished when shortening takes place under a load, for then a certain period of diffusion is allowed before the measurement of the speed is carried out, namely, the time between the immersion in the ATP bath and the development of the desired tension. In spite of this, the values of speed and also of power and efficiency, recorded in Table 10, even when recorded under a load, are only lower limit values. If we take into

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consideration the fact that the speed of the fibre model from skeletal muscle is measured both with a much higher load and at a far higher temperature than the speed of the muscle itself, it may be roughly estimated that the speed of shortening of the fibre model is at least ten times smaller than that of the living muscle.

Compared with this, the speed of shortening of the fibre model prepared from the slowest of all muscles on Table 10 (cow's rectum,

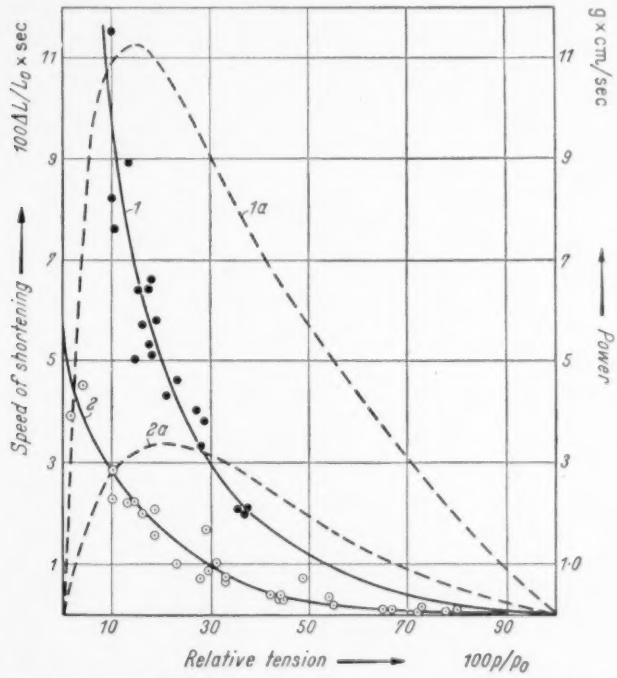

Fig. 15. Dependence of shortening speed and power on the load at 0°C and 20°C (G. and M. ULBRECHT[185]). Speed of shortening at 20°C (curve 1) and at 0°C (curve 2): refers to the left ordinate, power at 20°C (curve 1*a*) and at 0°C (curve 2*a*): refers to the right ordinate. Relative length 97 → 94 per cent p_0 is arbitrary for both curves = 1 kg/cm². All experimental points refer to the determination of speed

longitudinal muscle) seems to be relatively high: it appears to be only about ten times less than the speed of the model from skeletal muscle.

Exact measurements of the speed of shortening only exist in the case of the yellow adductor of *Anodonta* at 0° and 20° (G. and M. ULBRECHT[185]). Sufficiently thin fibre models can be prepared from this muscle, so that the ATP can reach the centre of the fibre. Unlike fibres prepared from skeletal muscles, these fibres do not tear even under a maximal isometric tension. Consequently, it is possible to keep the model in the ATP bath, in isometric contraction, until the steady state of diffusion inside the

model has been attained with certainty. Then the isometric tension is destroyed by a quick release, after which the tension starts to redevelop. As soon as it reaches the desired value, a limited isotonic shortening is allowed and its speed measured. At the end of this isotonic shortening the maximal isometric tension for the now resulting length is registered again.

The speed of shortening then depends on the load in an easily reproducible way if p/p_0 is used as a measure of load (p = tension of the

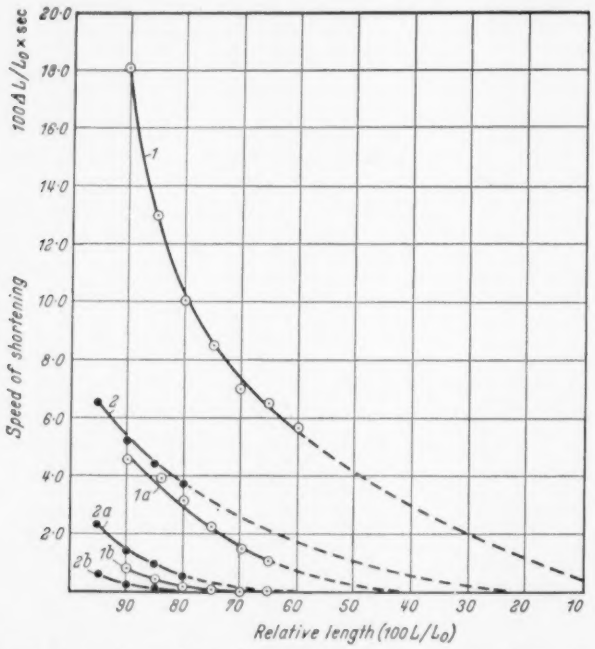

Fig. 16. The dependence of the shortening speed on the relative length of the fibre (G. and M. ULBRECHT[185]). Curve 1 (20°C) and curve 2 (0°C), with $p = 0$; curve 1*a* (20°C) and curve 2*a* (0°C), with $p = 0{\cdot}2\ p_0$; curve 1*b* (20°C) and curve 2*b* (0°C) with $p = 0{\cdot}5\ p_0$. p_0 = maximal tension at the relative length of 100 per cent

isotonic shortening in question, p_0 = mean value of the isometric tensions before release and after shortening). If the muscle without load shortens from 97 to 93 per cent of the resting length (see note under Table 10) the speed of shortening is 7 per cent of the length per second at 0°C, and 20–30 per cent at 20°C (Fig. 16). With increasing load it decreases at first steeply then more slowly (see Figs. 15 and 16). If an arbitrary value of 1 kg/cm² is inserted for p_0, and 1 cm³ is taken as volume of the model, the values of power given in Fig. 15 are obtained (curve 2 and 2a). In order to obtain the actual value of power in the individual experiment these values have to be multiplied by the values

of p_0 actually observed. The observed maximal tension p_0 is 1–1·6 kg/cm² at 0°C, whereas at 20°C it is 3–4 kg/cm². The ratio of power to the rate of ATP splitting represents the efficiency. Here the same rate of ATP splitting is taken as that with which a suspension of water-glycerol-extracted fibres splits ATP. In doing so it is supposed that the rate of splitting does not change during shortening. This assumption may be allowed, since the model fibre, in the absence of the M-B-factor, has largely lost the capacity of controlling the rate of ATP splitting. However, it is not certain whether the rate of splitting is constant during shortening, for the tetanized living muscle sets free 4–5 times more energy during maximal work in the unit of time than it does during

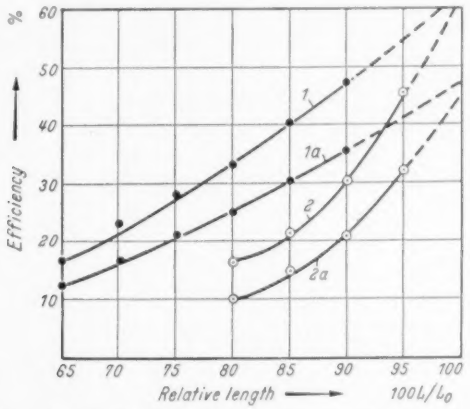

Fig. 17. The dependence of the maximal efficiency (with the tension $p = 0{\cdot}2\ p_0$) on the relative length of the fibre (G. and M. ULBRECHT[185]). Curve 1: average efficiency of 3 fibres with $p_{max} = 4$ kg/cm², curve 1*a*: average efficiency in all experiments with average $p_{max} = 3$ kg/cm². (Curve 1 and 1*a* at 20°C). Curve 2: average efficiency of 3 fibres with $p_{max} = 1{\cdot}6$ kg/cm², curve 2*a*: in all experiments with average $p_{max} = 1$ kg/cm². (Curve 2 and 2*a* at 0°C)

isometric tetanus (according to the experiments of the school of A. V. HILL,[1, 2, 4, 84] *cf.* further HILL[94a]). Should the model behave alike the efficiency given below would be much too high. The efficiency calculated in this way falls steeply as the shortening progresses, for the power diminishes with shortening by a decrease of the speed of shortening as well as by a decrease of p_0.

If the curves representing the efficiency (Fig. 17) are extrapolated to a relative length of 100 per cent, an efficiency of 50 per cent is obtained as mean value of all experiments. In the three experiments with the highest maximal tension, the mean value is approximately 65 per cent, independent of a temperature of between 0° and 20°C. The values are higher than those of the efficiency of the living skeletal muscle under anaerobic conditions. This may be due to the manner of calculation described above, or perhaps to the fact that the efficiency of the smooth

muscle may be higher than that of the skeletal muscle. Even if this should not be the case an unusually high efficiency would be plausible, since ATP splitting is the first link in the chain of reactions supplying energy; for it is improbable that the energy released in the different reactions which serve for the anaerobic restitution of ATP is applied for this purpose without loss. Consequently, the efficiency becomes less the longer the chain of restoring reactions. The efficiency of the whole anaerobic phase must necessarily be smaller than that of ATP splitting itself.

The high efficiency of the isolated working cycle in the best experiments shows that the low speed of contraction of models is largely caused by the small supply of energy. The rate of splitting of ATP during a single twitch of 1 g of living skeletal muscle is 6–8 μ Mol per second.* The comparable splitting rate by 1 g of fibre models from skeletal muscle (frog, rabbit) is only 0·6–0·8 μ Mol. Thus one tenth of the energy supplied for the contraction of the living muscle is available for the contraction of the model per second. Since the tensions produced in the contractions of muscles and models are equal, the speed of shortening must also be reduced to about $\frac{1}{10}$ if the efficiencies of both muscle and models are to be comparable.

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IV. Differences between the isolated working cycles and the working cycles of the various living muscles

The isolated working cycles resemble the working cycles of very differentiated living and intact muscles so closely that it will simplify matters to summarize the few discrepancies and evaluate their importance:

1. The models always have a definite rate of ATP splitting within the right ionic environment. Splitting by the models can only be prevented by the addition of inhibitors (see sections C, I*b*; C, II) or by removal of ATP.

The living muscle always contains ATP. In spite of this, during rest it splits practically no ATP at all. During the phase of contraction, however, ATP is split ten times more rapidly by the skeletal muscle than by its models (Table 12).

2. It can be inferred from the above-mentioned differences of the ATP splitting by living muscles and models that the models are, as a rule, contracted under the influence of ATP and in a rigid state without ATP, whereas the state of rest can only be produced artificially by the

* After about 70 contractions, a muscle poisoned by halogen acetate has decomposed its creatine phosphate completely and most of its ATP. It can therefore be calculated that 1 g of the muscle splits off 23 to 27 μ Mol. of phosphate during the contracting phases of these 70 twitches (LUNDSGAARD[114], [115]). At room temperature the whole period of these 70 contracting phases is approximately 3·5 seconds. The splitting rate of the fibre model can be calculated from the data of HASSELBACH,[74] SARKAR *et al.*[161] and PERRY[139] on the assumption that 1 g of fibre model contains about 0·1 g of protein.

addition of inhibitors (see sections B, II*a* and C, I*b*). The intact muscle, however, is able to vary its state between rest and contraction, although ATP is always present in it and it can do this without further chemical additions being necessary. Its content of ATP is seldom so small that it becomes rigid.

3. The speed of contraction of the models prepared from skeletal muscle is at least ten times less than that of the living muscle. This is shown in the preceding section concerning the speed of shortening. But the same seems to be true of the speed of development of tension (Table 11). The half-time values of models given on Table 11, indeed, are maximal values, as the experimental conditions of the experiments which are tabulated are not strictly isometric, moreover, the complications due to the diffusion of ATP have not been completely overcome.

TABLE 11

Speed of the redevelopment of tension after release to the tension 0
(Temperature 20°C)

System employed	*Animal*	*Half time value of the redevelopment of tension* (sec)	*Author*
Fibre model . .	Rabbit	18	A. WEBER[192]
	Anodonta adductor (yellow, white)	10	G. and M. ULBRECHT[184]
	Cow rectum (longitudinal muscle)	10	G. and M. ULBRECHT[184]
Actomyosin thread	Rabbit	15	PORTZEHL[144]
Tetanized muscle .	Frog (sartorius)	0·08	GASSER and HILL[61]
	Holothuria	4	A. V. HILL[81]

The differences in speed, which seem to be developed by different models, are of no significance, as they are partly based on the different initial lengths from which the release has been started.

The difference between the speed of contraction of smooth muscles and that of their models is less than the difference between the speed of living skeletal muscles and their models. By the isolation of the working cycle a certain levelling of the extremely diverging rates of the speed of the various kinds of living muscles takes place.

4. The maximal value of shortening is different for different kinds of living muscles. It fluctuates between ~ 80 per cent for some kinds of smooth muscles and ~ 30 per cent for certain skeletal muscles. The maximal shortening of fibre models, on the other hand, always amounts to ~ 80 per cent (Table 4). Thus the differences in the maximal shortening disappear completely when the working cycle is isolated.

These few differences between the behaviour of the living muscle and the models are relatively unimportant in considering whether the behaviour of the contractile protein in the two cases is based on the same fundamental process. For the differences in the conditions which bring about the transition from rest to activity can be traced back entirely to the differences in the conditions initiating the splitting of ATP in the muscle and in its model. The same applies to a great extent, and perhaps completely, to the dissimilarities in the speed of contraction (see section C, III, and further Table 11). This means that, under the conditions just discussed in paragraphs 2 and 3, the contractile protein in the model would in all probability act in just the same way as in the living muscle, provided the splitting rate of ATP were the same in both cases.

However, the difference in the extent of the maximal shortening of the skeletal muscle and its model seems not to be due to the difference of their rates of splitting, nor is it apparently based upon differences in the fundamental process of contraction. This dissimilarity disappears if the living muscle passes into the delta state (RAMSEY and STREET,[151] RAMSEY[150]). In the delta state the maximal shortening even of the living skeletal muscle amounts to ~ 80 per cent, and in addition to this its speed of shortening then falls to the low values obtained for the model.

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TABLE 12

Splitting of the optimal ATP concentration

Enzymatic system	*Breakdown of ATP* (μMol/mg *prot. min*)	*Author*
Fibre model (frog, rabbit)	~ 0·40	HASSELBACH[74]
Thread model (rabbit)	~ 0·35	HASSELBACH[73]
Fibre model (*Anodonta* adductor yellow)	~ 0·07	G. and M. ULBRECHT[184]
Fibre model (cow rectum)	~ 0·05	G. and M. ULBRECHT[184]
Living sartorius during contraction (frog)	~ 4·00	cf. note p. 98

As nobody will doubt that the fundamental process of contraction before and after the transfer of a living muscle fibre into the delta state is the same, in principle, then it must be concluded that the working cycle of all striated and smooth muscles and all models obtained from them depends on the same fundamental process. The latter is varied in the different kinds of muscles and models by variations in the amount of energy supplied to them in a given time by the splitting of ATP (see Table 12). In the scale thus resulting the skeletal muscle is first in its high level of tension and high velocity of shortening, whereas

the fibre model from cow's rectum, on account of its low tension and small speed of shortening, comes last.

However, it would be wrong to assume that the differences in the behaviour of the various muscles and models are dependent solely on the differences in the supply of energy. The peculiarity of the individual actomyosin controls what proportion of the power is determined by speed of shortening and what proportion by tension in the different muscles and models. The actomyosin of mammalian muscle develops the same tension both in the vital contraction with its high power and in the model contraction with its low power. The fibre models both from the yellow adductor muscle of *Anodonta* and from the skeletal muscle develop the same maximal tension, although the rate of splitting characteristic of the former model, and thus the supply of energy per unit of time, is seven times slower. On the other hand, the rate of splitting of the model from cow's rectal muscle is nearly the same as that of the model from *Anodonta*; nevertheless the tension produced by the former is ten times smaller (Tables 12 and 4).

The different actomyosins are functionally very closely related but not identical. This is shown by the fact that actin and L-myosin of quite different skeletal muscles of vertebrate animals—ranging from fish muscle to mammalian muscle—combine to give actomyosins with characteristic properties, more or less similar to those of the natural ones. The actin from vertebrate animals can even be replaced by the actin of spiny lobster. On the other hand, L-myosin obtained from spiny lobster is incapable of reacting with the actin obtained from vertebrate animals (CIGADA, CITTERIO, RANZI and TOSI[37]).

V. The regulation of the working cycle by ions and the influence of the Marsh-Bendall-factor

In the last two sections the variations in the working cycle of various muscles and models have been largely explained by variations of one fundamental process, brought about by regulation of the splitting of ATP. Now in the isolated working cycle, ATP is only split by actomyosin itself, see section C, I*b* and section C, II, (the living muscle contains some other ATPase apart from actomyosin). We have still to determine which *physiological* factors are involved in inhibiting and activating the ATP splitting and ATP contraction *in vivo*. Hence it seems particularly important to know how ATP breakdown and ATP contraction are prevented in the resting state and, on the other hand, activated after excitation.

The ATP concentration which inhibits both ATP splitting and contraction by virtue of being "over-optimal," varies according to the ionic strength. It decreases as the ionic strength increases (HASSELBACH[73]). Thus, even the physiological concentration of ATP may become

"over-optimal" if the ionic strength is high enough. However, the matter is complicated by the fact that the ionic strength at which this happens is also variable; for this critical ionic strength depends on the cohesive forces holding the actomyosin system together, and therefore on the concentration of actomyosin. The numerical ionic strength, which in the presence of the physiological ATP concentrations prevents both the contraction of the fibre model and the thaw contracture of the skeletal muscle is 0·4–0·5 μ (A. G. SZENT GYÖRGYI[179], [180] and A. WEBER[191]). The ATP contraction of the actomyosin thread with small protein content is inhibited by an ionic strength of $> 0{\cdot}1$ to 0·2 μ (PORTZEHL[142]). In fibre models which are rich in protein the critical ionic strength can be reduced to $0{\cdot}1 \rightarrow 0{\cdot}2\ \mu$ by treatment with plasticizers (pyrophosphate, alkaline bicarbonate solutions, A. G. SZENT GYÖRGYI[179], [180] and ROY[156]). This means that even with physiological ionic strengths, the physiological ATP concentration may be "over-optimal" under certain conditions and therefore cannot be utilized for muscle contraction.

Such conditions are found in the resting living muscle when the Marsh-Bendall-factor is present (MARSH[117], [118], BENDALL[18]). By means of solutions of low ionic strength (0·18 μ) this "factor" can be extracted combined with the easily soluble muscle proteins—at least in part. The crystalloids of the muscle can be removed from this protein solution by a twenty-four-hour dialysis against 0·15 M KCl + 0·01 M $MgCl_2$ + 0·001 M K-oxalate. The particle weight of the "factor" is accordingly higher than 10^4 (HASSELBACH[75]). The "factor" has so far not been isolated.

This "factor," activated by $5 \cdot 10^{-3}$ M Mg (BENDALL[19]), inhibits the contraction of the models or induces them to relax, as soon as the ATP concentration exceeds 1 to $4 \cdot 10^{-3}$ M, i.e. over the entire range of the physiological ATP concentration (HASSELBACH[75]). Under these circumstances it inhibits ATP splitting (Fig. 14).

The same solution of the "factor" inhibits both the breakdown of ATP and the ATP contraction less and less as the concentration of ATP is decreased (Fig. 14). A model soaked with the Marsh-Bendall-factor and treated with a physiological concentration of ATP contracts temporarily as long as the concentration of ATP inside the model is still low; it relaxes spontaneously when the ATP concentration inside the model has reached the level of the bath. If, during relaxation, a solution of the "factor" containing ATP is replaced by a solution of the "factor" free from ATP, the model contracts as soon as such an amount of ATP has diffused away so that its concentration inside the model is so low that the Marsh-Bendall-factor no longer acts. In this contracted state the model becomes rigid when the last trace of ATP has disappeared.

The inhibition caused by the M-B-factor is strongly influenced by the

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nature and concentration of the ions present: 1. It only occurs if the ionic strength is greater than 0·05 μ (BENDALL,[18] HASSELBACH[75]). 2. It is dependent on the presence of Mg ions and reaches its maximum when the concentration of Mg is higher than 5×10^{-3} M (HASSELBACH,[75] BENDALL[20]). 3. The inhibiting effect of the "factor" is reduced at a calcium concentration of 10^{-5} M and is lifted at a concentration of 10^{-3} M. This applies to breakdown (Fig. 18) as well as to contraction (MARSH,[118] BENDALL,[20] HASSELBACH[75]).

The glycerol-water-extracted preparations of fibres even without addition of the M-B-factor behave in approximately the same way as those to which the "factor" has been added, provided they are not more

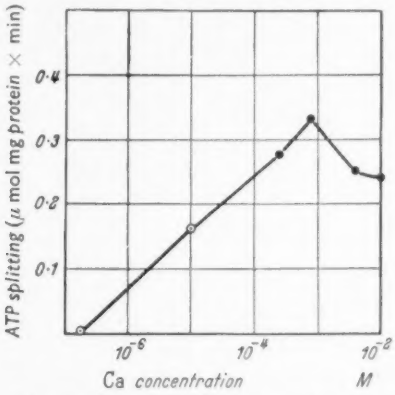

Fig. 18. The dependence of the ATP breakdown by the model fibre on the concentration of Ca ions in the presence of the M-B-factor (HASSELBACH[75]). 8×10^{-3} M ATP, 0·01 M Mg, pH = 7, ionic strength 0·2 μ ○–○–○ with addition of oxalate, concentration of Ca^{++} calculated from the solubility of Ca-oxalate. ●–●–● without oxalate

than three days old. In this case the "over-optimal" concentration of ATP (which cannot be applied for contraction) is slightly higher (15×10^{-3} M, BOZLER[28]). But all other phenomena resemble those described above. Particularly contraction occurs at once on addition of Ca (BOZLER[28]). Apparently fibre models which have not been extracted for too long still contain the M-B-factor in small quantitites.

As the M-B-factor does not render ATP unavailable in the contractile system by reacting with it, but by shifting the "over-optimal" concentration of ATP to lower values (Fig. 14), it must be concluded that the "factor" forms a complex with actomyosin. Mg causes relaxation because it activates the M-B-factor in this complex as inhibitor, whereas Ca produces contraction by inactivating the inhibiting factor (BOZLER[28]). In the isolated contractile (actomyosin) system the "factor" is absent, hence the same ions affect it quite differently.

Whilst the inhibiting effect of the "factor" is enhanced by increasing

ionic strength, the contraction of the actomyosin system without the M-B-factor is promoted by a decrease of the ionic strength. Since even comparatively high concentrations of ATP are still less than "over-optimal", when the ionic strength is not too high, it is possible to exchange indifferent ions such as K, Na, and the anions of the various buffers, as well as Cl, to a large extent without any noticeable change in tension and shortening. Investigations with radioactive isotopes have shown that the K ions of the muscles completely disappear if the water-glycerol-extracted fibre is treated with solutions which are free from K, but contain Na (HARRIS[71]). The fact that even under such conditions, in solutions which are free from K, contraction occurs with a maximal tension disproves all attempts to attribute to potassium an important role in the fundamental process of contraction (VERZAR,[190] FLECKENSTEIN[57]).*

K is evidently important in the excitation of the membrane before contraction takes place and perhaps at the moment when the excitation is transmitted from the membrane to the fibrils. The apparently widespread parallelism occurring in muscle between depolarization of the fibre membrane and the state of contraction, may denote that the excitation of the membrane and the active state of fibrils are electrically coupled (see below).

Obviously Mg alone is essential in contraction and cannot be replaced by other ions. This is striking in the case of contraction initiated by ITP. The latter is not capable of producing a superprecipitation of pure actomyosin or a contraction of fibre models unless a Mg concentration of $\cong 10^{-3}$ M is present (SPICER and BOWEN,[166] *cf.* BUCHTHAL and co-workers[33] with LAJTHA[108]). Nearly the same concentration of Mg is required for ITP to induce a dissociation of dissolved actomyosin (see SPICER and BOWEN[166]). Finally, the breakdown of ITP in the absence of Mg is only 20 per cent of that brought about when Mg is present (SPICER and BOWEN[166]). Ca activates splitting, too, but it is incapable of producing either superprecipitation and contraction of the gel or the dissociation of the solution (SPICER and BOWEN[166]).

With ATP all actomyosin systems contract, even without the addition of Mg, but more slowly and with a slighter development of tension. But all actomyosin systems actually contain traces of Mg (10^{-7} to 10^{-8} M, SPICER and BOWEN[166]) which cannot be removed by washing. However,

* FLECKENSTEIN has long been of the opinion that the storage of K in the muscle fibre even provides the energy of contraction by an interchange of K and Na. This theory had already been proved untenable before any observations on the isolated working cycle had been made. From the beginning its experimental basis was too slight (H. H. WEBER[198]). Moreover it is devoid of any experimental foundation, as it has been shown that the mechanical work of the muscle rises to a value 5 to 10 times larger than the amount of osmotic work of ionic interchange as soon as the muscle is sufficiently loaded. This fact may be mentioned, as it is published in a place which may easily escape notice (FLECKENSTEIN, HILLE and ADAM,[58] note 2 on page 279).

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if all traces of Mg are removed by washing with Graham's salt $(NaPO_3)_6$ or sequestrene, the actomyosin systems lose their capacity almost, if not quite, to split ATP or to contract with ATP and are unable to form superprecipitations or to dissociate in solution (H. H. WEBER[202], PORTZEHL,[145] HASSELBACH[73], [74]). An addition of Ca at once restores the splitting of ATP, whereas only the addition of Mg restores tension, shortening, superprecipitation, and dissociation besides splitting (HASSELBACH,[73], [74] PORTZEHL,[145] H. H. WEBER[202]). The reactions of ATP and ITP are altogether the same,* but ITP requires more Mg.

Mg cannot be replaced by alkali metal ions at all. It can be replaced by Ca ions only in activating the splitting of ATP.

All these facts offer a possibility of suggesting a link between the working cycle and the excitation of the membrane. Microscopic and electron microscopic experiments on ashed and evaporated material seem to denote that the electrolytes are not uniformly distributed but are localized in definite places (BARER,[14] DRAPER and HODGE[43], [44]). Especially, DRAPER and HODGE found that the alkaline earth metals lie in the fine cross bands which traverse the protein filaments of the fibrils at distances of 400 Å.

If we assume that Mg, thus localized in the fibrils, is bound with the M-B-factor, the muscle would be incapable of using its own ATP, as the inhibiting factor would be activated and the actomyosin inactivated. The muscle would then be at rest. In order to initiate a contraction the Mg needs only to move some 100 Å into the intervals between the bands in order to inactivate the M-B-factor and to activate simultaneously the actomyosin: this would represent the transition from rest to contraction. In order to terminate contraction Mg would have to travel back the same short distance. The effect would be intensified if Ca and Mg were arranged alternately in the muscle, so that Ca were bound to actomyosin in the resting muscle, but to the M-B-factor during contraction. For it is to be expected that Ca and Mg migrate when the membrane potential changes and migrate back when the membrane potential resumes its former value. This conception explains certain observations made by BARER[14] and FLECKENSTEIN (FLECKENSTEIN, HILLE and ADAM,[58] FLECKENSTEIN, WAGNER and GÖGEL,[59] FLECKENSTEIN, BROSE, CANIS and FÖRDERER[60]) concerning the close

* The results produced by the lack of the alkaline earth metals in the reactions with ATP cannot be misinterpreted. They are not caused by a poisoning effect of the softening agents. This is proved not only by the similar behaviour of the ITP reactions, but also by the fact that the addition of Ca restores only one, whereas an addition of Mg restores all ATP reactions, although the softening agents are bound by both ions. The totally different constitution of Graham's salt and aethyl-diamino-tetra-acetate is a further argument against an indentical poisoning effect of these softening agents. On the other hand, even fluorides, which also form complexes with Mg, at a concentration of 0·1 to 0·2 M, inhibit the contraction only when the concentration of ATP is kept low (2×10^{-3} M).

coupling between the duration and the intensity both of the depolarization of the membrane and of the contraction of the fibril.

Secondly it explains the extraordinary rapidity (A. V. HILL[88]) with which the contraction of the fibrils follows on the changes of the polarization of the membrane; for when the electric field changes both the earth alkaline ions in the depths of the fibres and the alkali metal ions in the membrane would begin to move at the same instant. The distance, however, to their new scene of action would be short (see above).

Thirdly, the hypothesis explains the continuous ATP contraction of the actomyosin models minus "factor" and the continuous relaxation of the models with active "factor": this is due to the fact that the models have no membrane, the changing polarization of which would alternately activate and inactivate the inhibiting "factor" and the actual contractile system.

The facts recorded above appear to be practically certain, but the attempt to connect the working cycle with the events of excitation is more or less speculative.

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D. General Conclusion

The observations which have been discussed can be summed up in the following description of the working cycle of the living muscle.

1. The energy of the energy-rich phosphate bond is not transmitted to the contractile system during rest, because it has been shown that the splitting of ATP does not take place at that time. During rest, ATP itself is bound to the actomyosin, and acts as a plasticizer in rendering the fibrils very extensible and almost plastic (section B, II*a*).

2. The breakdown of ATP is inhibited during rest, owing to the active state of the Marsh-Bendall-factor. To be active two conditions must be fulfilled: the "factor" must be bound to Mg; the "factor" must not be bound to Ca. It obviously remains in this state as long as the membrane potential of the fibre is high (section C, V). However, when the resting potential diminishes sufficiently, or changes its sign, the Mg ion is transferred to actomyosin while the "factor" combines with Ca. The M-B-factor then becomes inactive and the actomyosin active. The splitting of ATP and contraction now occur (section C, V).

3. During a single twitch the change of polarization is great but only lasts a short time. Therefore the breakdown of ATP and contraction are of maximal intensity (A. V. HILL,[86], [91]) but quickly finished. On the other hand, during reversible contractures the intensity both of the contracture and of the breakdown of ATP is less; but they take more time due to the fact that the change in the polarization of the membrane is also smaller and takes time; for during reversible contractures the membrane potential is merely lowered without a change in sign.

4. When the splitting of ATP ceases without a large decrease of the level of ATP the muscle relaxes (section C, I).

5. If the content of ATP is practically exhausted rigor sets in (section B, II*a*, *b*). Rigor is often accompanied by contracture, as the M-B-factor—even in an activated state—is capable only of preventing the breakdown of physiological concentrations of ATP but not the splitting of very small ones (section C, II). If the decomposition of the residual ATP takes place rapidly enough the muscle becomes rigid in contracture.

6. The energy of metabolism is transmitted to actomyosin during the contracting phase of the working cycle, whereas the phase of relaxation is the thermodynamically spontaneous part of the cycle (section C, I). The energy transferred is the energy of the splitting of ATP. So far as has been measured this is transformed into mechanical work with a very high efficiency (section C, III).

7. The transfer of energy during contraction does not depend on a change in the axially parallel alignment of the contractile molecules but on a change in their shape (section B, III*b*; see also ASTBURY[7]). The mechanism of this transformation is as yet unknown.

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3

THE SUBMICROSCOPIC STRUCTURE OF NERVE FIBRES

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EVER since their discovery nearly three centuries ago, nerve fibres have been studied with undiminished interest by succeeding generations of microscopists, largely because of the remarkable properties of such apparently simple structures. For although these principal conducting elements of the nervous system exhibit complex physiological and electrical properties, they appear in the living state merely as delicate threads of protoplasm with few discernible structures. Attempts to analyse their composition are seriously hampered by the labile organization of nerve protoplasm which is extremely sensitive to the action of chemical and physical agents. Moreover, the limited resolving power of the light microscope has failed to reveal the expected complexity of fine structure, even after subjecting the nerve fibres to the modifying treatments of histological techniques. It was therefore natural to seek after the physical substratum for impulse propagation and other aspects of nerve activity at the lower submicroscopic level of molecular organization.

The long series of investigations leading to our present knowledge of nerve ultrastructure started one hundred years ago with EHRENBERG'S[31] discovery of the birefringence of nerve. Extensive polarized light studies of this effect demonstrated that the myelin sheath had a highly ordered structure of fluid-crystalline nature. A more detailed picture of the layered structure in the myelin sheath was then derived from x-ray diffraction studies, establishing the existence of a highly organized structure which only lacked the ultimate confirmation of direct visualization. Thus, when the electron microscope was finally applied to the study of nerve fibres, the submicroscopic components predicted by these indirect methods could be directly demonstrated and correlated with the information already available. Beyond this, electron microscopy has made it possible to follow the fundamental membrane and filamentous structures throughout the whole range of nerve fibre size, down to their simplest association as single components of submicroscopic elements. In fact, the analysis of nerve fibre organization offers one of the most instructive examples of how the fine structure of a biological object can be systematically unravelled by the combined use of indirect and direct methods.

However, the extreme lability of nerve tissue has also made it a

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classical object for the production of artefacts. With this in mind, the effects of the preparation techniques employed in electron microscopy must be carefully evaluated, since they involve fixation, dehydration, embedding and sectioning of the object, to mention only a few of the factors. Every opportunity must be taken to refer the observations to the structure of the living object. It is therefore important that the results of polarized light and x-ray diffraction studies which were performed on fresh nerve without introducing significant alterations, are in satisfactory agreement with the electron microscopical findings.

The present account will deal mainly with the results obtained so far in the examination of a wide variety of peripheral and central myelinated nerve fibres, and certain types of unmyelinated fibres with the electron microscope. Salient features of relevant investigations with the light microscope, polarized light, x-ray diffraction and other methods are reviewed first as a necessary background for the electron microscopical studies.

Nerve Fibre Structure as Revealed by the Light Microscope

When examined in the living state[130] or isolated under conditions not impairing their normal functional activity[61], [79], vertebrate myelinated nerve fibres appear as long cylindrical threads, usually 2–20 μ in diameter. The individual fibre consists essentially of a cylindrical core or axon within a glistening tube of myelin, which is covered with a thin membrane and appears interrupted at periodic intervals of 1–3 mm by circular constrictions or Ranvier nodes. The myelin sheath is separated by these constrictions into internodal segments, which in view of the regularly associated Schwann nucleus were already regarded by Ranvier[93] as characteristic cellular units. Inside each segment the myelin is divided by oblique clefts, the incisions of Schmidt-Lantermann which have also been observed in isolated living fibres,[79] into portions of cylindro-conical shape. Except for these constantly discernible structures, the sheath and axon of normal fresh fibres show no further microscopic details in visible light,[23] under dark field examination[36], [37] in polarized light[104] and also in ultraviolet light according to Massazza.[75] However, Muralt[79] has observed a fine network which is particularly distinct near the nodes in isolated fresh nerve fibres examined with short wavelength (2570 Å) ultraviolet light. The axon of fresh vertebrate fibres is usually homogeneous, but under certain conditions characteristic fibrillar structures, the neurofibrils, can be observed in nerve tissue cultures.[73], [136] Well defined threadlike neurofibrils have also been demonstrated in living invertebrate nerve fibres,[15], [24] where they form a central longitudinal bundle and are surrounded with viscous, unfibrillated axoplasm. Without having to

apply histochemical techniques, much can be learned about the relationship and properties of the main components of nerve fibres by micromanipulation. As a basis for micromanipulation experiments, DE RÉNYI[23] established that a relation between the diameters of the axis cylinder and the myelin sheath corresponding to a ratio of 1 : 1 in cross-sectional areas is characteristic of normal living frog nerve fibres. By injecting saline solution with a micro-pipette into the fibre its three components, axis cylinder, myelin and the adherent neurilemma membrane can be easily separated from each other. Myelin is organically connected to the axon, but can be pulled off without altering the shape of the axis cylinder. However, an axolemma membrane bounding the axon does not seem to exist. The substance of the axon is considered to be of "soft jelly-like consistency",[23] also plastic and elastic to a certain extent. The axon is constricted at the nodes, where its substance appears to be more viscous or solid than in the internodes. Recent micromanipulation experiments performed by SCHNEIDER[121] on isolated frog nerve fibres show that they can be stretched up to 30 per cent at the internodes without abolishing impulse conduction. Although nerve fibres are very sensitive to direct injury, they can be isolated in an apparently normal state from the surrounding connective tissue because of this tolerance to longitudinal stretching.

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Observations based on experiments with nerve fibres which have been constricted[135] or cut[137], [138] indicate that there is a continual flow of material from the nerve-cell body along the axon, with fluid loss through the surface. According to YOUNG[139] this pressure maintains the organization of the substance of the axoplasm, and the axon breaks up into sections if it is removed. This author[139] has also pointed out the important influence of surface tension forces and other physical factors in determining the structure of myelinated nerve fibres. In contrast to the simple relations observed in fresh material, fixed and stained nerve fibres reveal a variety of more complicated structures.

Each nerve fibre is then clearly seen to be covered with a very thin, structureless membrane generally called the neurilemma or Schwann's sheath, which constitutes the inner edge of the tube wall surrounding the fibre. According to PLENK[89] this tube wall consists of two layers, an inner endoneurium or sheath of PLENK and LAIDLAW,[67] and an outer endoneurium referred to as the sheath of KEY and RETZIUS.[63] The inner endoneurium is formed by a delicate network of circular argyrophil fibres, while the outer endoneurium consists of collagenous longitudinal fibres. The latter is continuous with the external fibrous layers closely surrounding the nerve fibre and known as Henle's sheath. The exact nature of the neurilemma membrane and its relation to the Schwann cell protoplasm, which appears to cover the whole segment, still remain to be determined. The perinuclear cytoplasm of the

Schwann cell contains several types of granulations[91] which are differentiated by their affinity for certain reagents, like the granulations of REICH[94] and the fatty corpuscles of ERZHOLZ.

The substance of the myelin sheath presents different aspects according to the applied histochemical treatment. After fixation with potassium bichromate the sheath separates into concentric lamellae, but lipoidal rodlets arranged radially in the sheath have also been described. A reticular framework, the neurokeratin net, remains after extracting the lipids from the sheath. In fresh nerve fibres stained with methylene blue a spongy framework resembling the neurokeratin net is also seen within the myelin sheath. Special spiral structures, the threads of GOLGI-REZZONICO, or a granular cementing substance are seen inside the funnel-shaped incisions after appropriate staining.[90]

The details of the neurofibrillar structures differentiated in the axon[2], [90] depend likewise on the type of fixation and staining, which undoubtedly determine certain artificial aspects of these formations. It is assumed, however, that there are at least some regularly arranged, long submicroscopic particles in the axoplasm which may aggregate under suitable conditions to give visible neuro-fibrils. Interferometric analysis[68] of fresh nerve fibres indicates that a submicroscopic spacing of about 0·3 μ exists between elements oriented parallel to the axis of the axon. This fine structure is replaced by visible fibrillar formations upon staining.

Certain characteristic annular structures like the double axonic bracelet of NAGEOTTE[80] have been described at the nodes. The so-called disc of Ranvier is a plate or diaphragm located between the two parts of this spiny bracelet, through which the constricted axis cylinder passes. MURALT[79] also considers the presence of a transversal membrane with pores or a space where substances can pass at the nodes in the living nerve fibre.

Despite the multitude of described structures, the fact remains that light microscopy has not succeeded in solving such fundamental problems as the relation of the myelin sheath to the axon and neurilemma, or even clearly determining the structure of any of these components.

POLARIZED LIGHT STUDIES OF NERVE

Polarized light studies of nerve have played a decisive role in disclosing the ultrastructure of its components, and the reader is referred for detailed reviews to the papers by GÖTHLIN,[55] SCHMIDT,[104] SCHMITT and BEAR,[114] and MURALT.[79] When viewed under the polarized light microscope the strong birefringence of the myelin sheath predominates over the weak positive uniaxial birefringence of the axon. The myelin embedded in the sheath produces this birefringence, which is positive referred to the radial direction and reaches values up to 0·011 in A

fibres of frog sciatic nerves according to quantitative determinations made by SCHMITT and BEAR.[113] Compared with the figure of 0·023 obtained for pure ammonium oleate crystals, this value indicates a high degree of orientation of the anisodiametric, optically positive lipid molecules, which are considered to have their optic axes directed radially in the sheath. Myelin extruded from the sheath shows the same microscopic appearance and optical properties as the characteristic tubular myelin forms produced by the action of water on mixed or pure lipid components extracted from nerve. If the lipids are extracted from the sheath by treatment with alcohol the magnitude of the natural birefringence is markedly reduced and its sign reversed, thus demonstrating the importance of the lipid components in determining the optical properties of the myelin sheath. The remaining framework of protein contributes the negative uniaxial birefringence which is responsible for the reversal of birefringence.[76], [104], [20] According to immersion experiments, the negative protein birefringence is mainly textural birefringence with optic axes directed radially as before extraction. Since this would correspond to anisodiametric particles with their long dimensions arranged parallel with the surface of the sheath according to WIENER's theory, it has been assumed that the proteins are organized as platelets or lamellae.[104], [20] This assumption finds support in the tendency of the fixed myelin sheath to cleave into concentric lamellae.[80] However, the appearance of the protein residue as a fine or coarse meshed network depends largely on the way the lipids are extracted from the sheath.[104] The protein of the sheath was characterized as "neurokeratin"[65] based on solubility and digestibility data, but BLOCK[13] questions this classification and infers that the neurokeratin may not even be located in the myelin sheath. Since so little is known about the chemical nature and localization of the sheath proteins it is impossible to determine how they are arranged in the postulated concentric layers.[110], [112]

Earlier investigators[64], [65] ascribed the birefringence of the sheath to the presence of oriented lipid fluid-crystals in the myelin. With succeeding advances in the polarized light analysis of the composite lipid protein structure of the myelin sheath, it became possible to arrive at a more detailed picture of its organization. Thus SCHMIDT[104], [105] deduced from polarized light studies that the myelin sheath is essentially built up of concentrically arranged protein lamallae alternating with lipid layers (Fig. 1). This structure was confirmed by x-ray diffraction studies and serves as a basis for all subsequent work on the organization of the sheath.

Polarized light investigations[55], [9], [10], [20] also show that the thin sheaths of invertebrate nerve fibres are fundamentally similar to the myelin sheaths of vertebrate fibres, differing mainly with respect to

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the relative amounts of oriented protein and lipid present.[9], [114] Fixation of the myelin sheath with osmium which is used extensively in preparing specimens for electron microscopy, modifies the sheath birefringence in a characteristic manner. According to SCHMIDT[104] osmium fixation abolishes the birefringence of the lipids. But the black reaction product of osmic acid (osmium dioxyhydrate) is deposited in an oriented way in the myelin sheath, introducing a weak positive uniaxial birefringence with radially oriented axis which partially com-

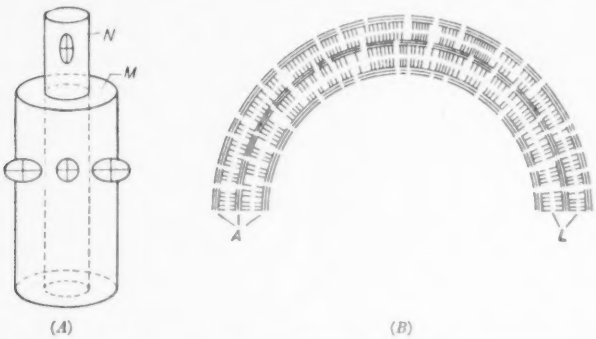

Fig. 1. Schematic diagram of medullated nerve fibre structure based on polarization optical analysis. (*A*) N = axon positively uniaxial as referred to axial direction. M = myelin sheath positively uniaxial as referred to radial direction. (*B*) Submicroscopic structure of the myelin sheath (after SCHMIDT). (After FREY-WYSSLING[49])

pensates for the loss of lipid birefringence. Osmium fixation is therefore suitable for preserving lipid structures, but it also introduces oriented inclusions and favours the formation of water layers between the components of multilayered systems.[106]

Polarized light studies indicate that there is a remarkable similarity between the structure of the myelin sheath and that of the outer segments of the retinal rods. This has led SCHMIDT[106] to compare a rod outer segment with a radial cylinder of the myelin sheath. The rod outer segments show positive uniaxial birefringence which reverses its sign upon extraction with lipid solvents or fixation with osmium. SCHMIDT[102] has demonstrated that the resulting negative uniaxial birefringence is a platelet form birefringence. From polarized light analyses and complementary dichroism studies SCHMIDT[103] deduced that the rod outer segments consist of transversally oriented submicroscopic protein layers alternating with layers of longitudinally oriented lipid molecules. This is therefore fundamentally the same type of laminated structure that was postulated for the myelin sheath and could be confirmed later by direct observation with the electron microscope.[125]

The axons of fresh vertebrate nerve fibres show weak positive uniaxial birefringence,[55], [104] but detailed studies are difficult to perform because of their small size and unfavourable location within the myelin sheath. The axons of fresh squid giant fibres are more suitable for quantitative polarization optical studies,[11] which have definitely demonstrated the presence in fresh axoplasm of anisodiametric micelles with intrinsic crystalloidal structure. From this analysis, BEAR, SCHMITT and YOUNG[11] have deduced that squid axoplasm contains a relatively small fraction of well oriented elongate particles having widths small with respect to the wavelengths of light. These particles must have considerable regularity of internal structure as judged by the intrinsic birefringence, and they are oriented parallel with the fibre axis. It is assumed that these components may correspond to the submicroscopic filaments[109] or the lattice responsible for the formation of neurofibrils under certain conditions. Giant axons of the squid are also a favourable object for investigating the chemical properties of the axoplasm's protein components. A protein complex called neuronin[12] was hereby isolated which seems to be characteristic of neuroplasm generally, and exhibits solubility properties of a nucleo- or pseudonucleoprotein. The maintenance of the normal state of aggregation of this protein complex depends largely on the *p*H and on the presence of dissolved salts.[12]

X-RAY DIFFRACTION STUDIES OF THE MYELIN SHEATH

In order to obtain more detailed information about the configuration and dimensions of the highly ordered molecular structures revealed by polarized light analysis, x-ray diffraction studies were performed on nerve.

A significant advantage of this method is the possibility of working with undamaged whole nerve under physiological conditions without having to isolate single fibres, since the recorded diffraction pattern of fresh nerve corresponds very nearly to "that which would be obtained from an internodal segment of a single nerve fibre".[114] It has also been ascertained that the amount of radiation necessary for recording the diffraction patterns seems to have negligible effects upon the action potentials and irritability of nerve.[115], [32] Most of our present knowledge on the organization of the myelin sheath as derived from x-ray analysis is due to the classical investigations of SCHMITT, BEAR and CLARK,[115] and SCHMITT, PALMER *et al.*[116], [119], [8], [84] The pioneering work of HANDOVSKY and THIESSEN[56] and BOEHM[14] was more exploratory in character, and only recently have new contributions been made in this field by ELKES and FINEAN.[32], [33], [46]

The essentially similar patterns obtained from a variety of fresh vertebrate myelinated nerves can be attributed entirely to the myelin

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sheath components, since they are nearly completely reproduced by benzene extracts of nerve rubbed with water.[115] The clue to the interpretation of the myelin sheath structure was furnished by the long-spacing diffraction patterns. Characteristic long-spacing equatorial diffractions were observed which appeared to be the first four orders of a fundamental spacing of 171 Å in amphibian nerves (Fig. 3*a*) and of 184 Å in mammalian nerve.[115], [116] However, the possible existence of even higher unit spacings is conceded,[115] which may be revealed with improved methods. When nerve is dried, a reduced unit spacing of 144 Å (amphibian) and of 158 Å (mammalian) can be recorded directly. In dried mammalian nerve the patterns also show three shorter spacings at about 63·5, 44 and 34 Å, which are identical with the spacings found in dry total lipid extract of nerve.[119] It will be recalled here that the lipids extracted from the nervous system comprise the phospholipids (lecithin, cephalin and sphingomyelin), cerebrosides (phrenosin, kerasin, nervon, oxynervon) and the sterols (mainly cholesterol and its esters).[133], [83], [19], [48] These lipids give intense long-spacing x-ray diffractions, with values corresponding to the length of their characteristic bimolecular leaflets. Extensive x-ray studies have been carried out on nerve lipids in single and mixed systems[8], [84], [116] and on lipo-protein complexes.[85]

These studies have shown that the fundamental long-spacing of fresh or dried nerve cannot be duplicated with lipid mixtures or lipid emulsions, which upon drying only give the spacings characteristic of the double layers of the separate phases of lipids. Moreover, the patterns of fresh nerve show a marked alternation in the intensities of the long-spacings assigned to the even and odd orders of the fundamental period, which is not seen in the patterns of dilute emulsions of mixed lipids containing no protein.[84], [119] From this diffraction evidence it is concluded that "only the presence of protein in the repeated fundamental structure of the myelin sheath can account for the 140–160 Å period of dried nerves".[116] Apparently, the inclusion of neurokeratinogenic protein, probably in the form of intercalated concentric lamellae suggested by polarized light studies, is needed to build up the complex units of the sheath. Interpreting the long-spacing equatorial diffractions in terms of the sheath's radial organization, it was therefore assumed that the fundamental period corresponds to the thickness of the concentric lipid-protein layers deduced from polarized light studies.[115], [116] The meridionally accentuated short spacing at 4·7 Å recorded from fresh nerve would correspond to the random distribution of the radially oriented lipid molecules in the tangential direction, indicating the average interchain separation of their hydrocarbon chains. The layers have the properties of the smectic fluid-crystalline state, since the lipid molecules which are constrained with their

hydro-carbon chains oriented perpendicular to the planes of the layers, still have freedom of rotation and translation within these layers.[112]

The specific myelin structure associated with the long-spacing identity periods must be relatively stable, since the spacings and the orientation of the equatorial spots are unaltered by heating nerves to 70°C, soaking in detergents, isotonic KCl or $CaCl_2$.[116], [119] Based on the available polarized light and x-ray diffraction data SCHMITT *et al.*[114], [116] consider that the living myelin sheath is of "smectic mixed-fluid crystalline nature and consists essentially of lipid-protein layers about 180 Å thick wrapped concentrically about the axon" (Fig. 2). Assuming that the

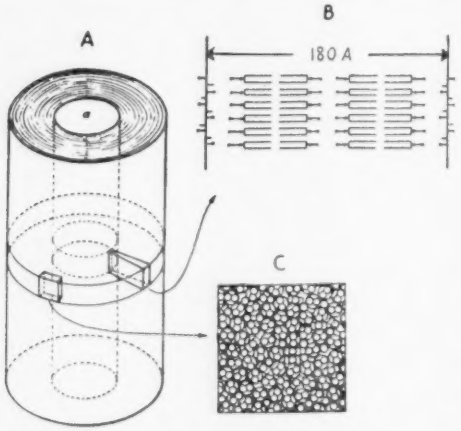

Fig. 2. Schematic representation of myelin sheath structure showing: (*A*) Concentric lipid-protein layers. (*B*) Structure in the radial direction (perpendicular to the planes of the layers). (*C*) Structure in the planes of the layers (paraffin chains of lipid molecules shown as open circles). (Figure kindly provided by Prof. F. O. SCHMITT)

identity period of mammalian nerve contains approximately 134 Å of mixed lipids and 25 Å of protein, these authors[116] discuss several possible ways of arranging the coaxial lipid and protein layers in the myelin sheath. The protein may occur as single or multiple monolayers between each double layer of lipid or between alternate double layers. In order to explain the long-spacing identity period, differences in geometrical arrangement between the two lipid bimolecular leaflets in the structural unit must also be assumed.

All other attempts to pursue the analysis of myelin sheath structure further are hampered by the lack of direct quantitative data on its water content and composition with respect to protein and lipids. Only a part of the sheath's water content, estimated to be at least 35 per cent, can be localized in the identity period. The existence of micelles or concentric slabs of myelin separated by thick channels of aqueous

phase[116] is therefore considered. Most of our information about the quantitative distribution of lipids and protein is still derived from analysis of whole nerve, and it will therefore be necessary to carry out such determinations directly on single nerve fibres. A beginning in this direction are the extraction experiments with fat solvents performed on single nerve fibres by ENGSTRÖM and LÜTHY[35] using the x-ray absorption technique for the determination of mass. These studies indicate that the extractable lipids constitute about 50 per cent of the central part of the myelin sheath. A detailed investigation of the chemical properties of the neurokeratogenic protein is also essential to a better understanding of myelin sheath structure.

Since the entire x-ray diffraction pattern of fresh nerve is due to its lipids which are predominantly located in the sheath, no definite information has been obtained by this method on the organization of the axon. According to SCHMITT[107] the proteins are represented only by the 10–13 Å spacing in dried nerve, but it cannot be determined whether this is due to the connective tissue or partially also to the axon proteins.

Recently ELKES and FINEAN[32], [33] have studied the effects of drying, freezing and thawing, and treatment with lipid solvents on the structure of the myelin sheath of frog sciatic nerve by means of x-ray diffraction methods. The recorded long- and short-spacing diffraction patterns are in general agreement with the results obtained by SCHMITT, BEAR and CLARK,[115], [116] and are interpreted by ELKES and FINEAN in terms of the proposed normal myelin structure consisting of alternate layers of lipid and a lipoprotein material arranged concentrically around the axon. According to these authors, there is a "labile" lipid component in this lipid-lipoprotein system which separates readily from the normal structural unit, forming poorly oriented separate phases, and leaving a still highly ordered "residual complex" containing more tightly bound lipid molecules. It was furthermore noted that the experimental modifications of structure apparently lead to increases in the principal spacings of the diffraction pattern, compared with those obtained from fresh normal nerve (Figs. 3, 4). The "residual complex" spacings of nerve modified before drying are likewise higher than the corresponding spacings recorded from normal dried nerve.

In a detailed analysis of the x-ray diffraction data, FINEAN[46], [47] tries to account for the increase in spacing of the order of 20–30 Å which appears to result from the described treatments, by considering an expansion in layer thickness and changes in the organization of the lipid layers. Thus, the expansion of the residual complex spacing can be explained by considering variations in the angle of tilt of the lipid molecule within the bimolecular leaflets of each layer. Changes in spacing of more than 25 Å observed among the polymorphic forms of

isolated phospholipids[46] and in some of the fractions of total nerve lipid extract at different temperatures[34] have also been ascribed to such variations in the angles of tilt of the long lipid molecules.

Fresh nerve which is dried slowly shows a residual complex spacing of 73·5 Å probably representing a second order diffraction, while the fainter first order of 148 Å can be recorded directly. In this case it is possible to conceive that the differences in organization of the alternate bimolecular lipid layers comprising the structural unit are due to variations in the direction of tilt of the lipid molecules, in such a way that "adjacent lipid layers are approximately mirror images of each other"[46]. However, in nerves modified (i.e. frozen and thawed) before drying the residual complex spacings of 95–100 Å appear to be the fundamental spacing, since no higher spacings are recorded.

This would mean that the unit cell need only contain one bimolecular leaflet instead of two, which can be explained by assuming that adjacent lipid layers have become identical because the lipid molecules are no longer tilted but appear instead arranged perpendicular to the plane of the leaflet. The dimensions of 90–100 Å for such a structural unit, presumably representing the thickness of the single layers in modified nerve, are of particular interest in connection with the results of electron microscopy to be discussed later. In this connection, preliminary studies of the effects of osmium fixation on the x-ray diffraction pattern of nerve have shown that the precise layered structure of the myelin sheath is preserved. The recorded low-angle diffraction patterns of osmium fixed nerve are well orientated and indicate a change in layer thickness as compared with fresh myelin. It would seem that the osmic acid also tends to bind the lipids more firmly in the layered structure.

These conceptions derived from studies on dried nerve can also be applied in the interpretation of the fresh nerve pattern (Fig. 3*b*). Since the fundamental repeating unit of 170 Å in fresh frog nerve probably consists of two protein layers 30 Å thick and two layers of lipid approximately 55 Å each, the "longest lipid molecules must be curled or tilted in some way in order to accomodate them in the layers".[47] The radial orientation deduced from polarized light and x-ray diffraction studies would favour the curled form. Considering ways of stabilizing these curled lipid molecules within the layers, FINEAN[47] offers the interesting suggestion of a stable complex formation between cholesterol molecules and the phospholipid or cerebroside molecules. Cholesterol is found in myelin only as free cholesterol in approximately equimolecular proportions with phospholipid. In the proposed arrangement, each long-chain lipid molecule is stabilized individually by a cholesterol molecule to give the required bimolecular leaflet of about 50 Å.

While these considerations are still largely speculative in character, they fit in well with the available x-ray diffraction data. The possibility

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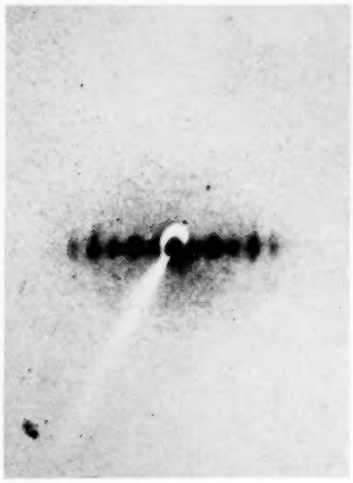

Fig. 3*a*. Small-angle pattern of fresh nerve (green frog sciatic) L = 16·88 cm. (Figure kindly provided by Prof. F. O. SCHMITT)

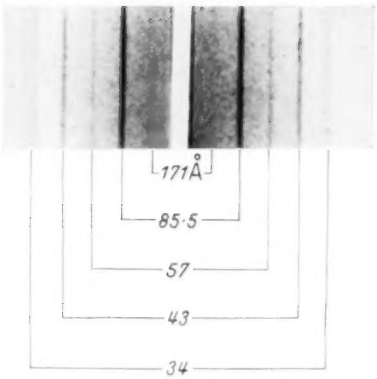

Fig. 3*b*. Low-angle diffraction pattern of fresh frog sciatic nerve

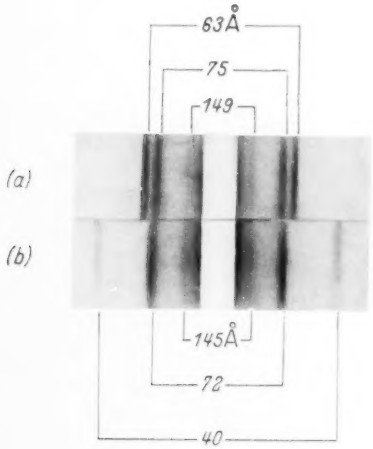

Fig. 4*a*. Example of change in low-angle diffraction pattern produced by heating. (*A*) Ether-treated dried nerve at 20°C. (*B*) Same specimen at 100°C

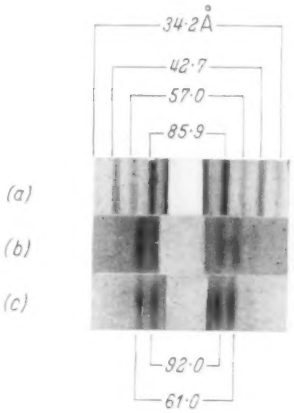

Fig. 4*b*. Low angle diffraction patterns of nerve obtained at different temperatures. (*A*) At 20°C. (*B*) At − 2°C. (*C*) At − 40°C. (Patterns 3*b*, 4*a*, 4*b* obtained using slit collimating system at 10 to 20 cm sample-to-film distance. All figures kindly provided by Dr. J. B. FINEAN)

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is also illustrated of arriving at a more detailed picture of myelin ultrastructure by correlating x-ray diffraction studies of experimental modifications in nerve with what is known of the molecular structure of its individual components. Further progress will therefore mainly depend on obtaining more precise information about the chemical composition and quantitative distribution of the different lipid and lipoprotein constituents of the myelin sheath.

Electron Microscope Studies of Nerve Fibres

The electron microscope's greater resolving power was not only expected to reveal the highly ordered structure deduced from the foregoing investigations, but also to elucidate the details of nerve fibre organization. Successful application of this instrument to the study of nerve fibres depended on the development of adequate preparation techniques capable of yielding ultrathin sections which exhibit satisfactory integrity and preservation of fine structure. Most of the methods now available for ultrathin sectioning of tissues[16], [81], [86], [127] require embedding of the material in collodion-paraffin or methacrylate, involving preliminary dehydration with alcohol-ether which may affect the lipid constituents of nerve. In order to avoid these embedding treatments a technique was developed[42], [42] which makes it possible to prepare ultrathin serial frozen sections of unembedded fixed or fresh nerve. This method proved to be particularly suitable for the examination of a small, circumscribed object having a compact layered structure like the nerve fibre. For the absence of an embedding matrix with its agglutinating effects greatly facilitates the analysis of the fibre's concentric layers of submicroscopic membranes and filamentous structures.[39], [40] A significant advantage is also the possibility of working directly with thin fresh sections of nerve which can be fixed or frozen-dried by a special procedure.[42], [44] Moreover, the well-preserved nerve fibre sections can be subjected in the fresh state to enzymatic digestion, microincineration and different cytochemical reactions, and the effects followed with the electron microscope. In view of their tenuity the sections are also ideal objects for parallel studies with the phase contrast and polarized light microscopes.[44] In the small areas required for electron microscopy, the degree of preservation of fine structure exhibited by the thin frozen sections is satisfactory, but structural relations involving larger portions are not well preserved.

It is therefore always necessary to examine ultrathin sections of embedded material, and many nerve fibre studies[30], [87], [99] are based exclusively on this type of preparation. With recent improvements in the preparation and sectioning of embedded material,[128] certain fine structures can now be clearly resolved throughout the whole specimen which previously could only be detected in limited areas of frozen

sections. But no single method, however refined, can be relied upon to give a complete picture of the nerve fibre's structural complexity. This can only be obtained by applying a combination of numerous complementary methods, including dissociation procedures for the isolation of single elements.

The studies to be reviewed here deal chiefly with the structure of the myelin sheath and the axon at the internodes, since comparatively few observations of the node's organization have been recorded.

The Structure of the Myelin Sheath

The myelin layers

The first attempts to demonstrate the postulated layered structure of the sheath by electron microscopy[124], [110], [39] were carried out on fragmented preparations of nerve. Thin membranes were isolated from fragmented, osmium-fixed peripheral nerve which according to SJÖSTRAND[124] and SCHMITT[110] may have been derived from the myelin sheath, but could not be adequately localized with the fragmentation technique. Similar lamellar structures were also observed by FERNÁNDEZ-MORÁN[39], [40] and could be definitely localized within the myelin sheath by examining thin frozen sections of osmium-fixed myelinated nerves. In a series of investigations[40], [44] this author has studied the main features of the myelin layers.

In thin frozen sections it is seen that the myelin sheath dissociates into thin concentric layers which are identical with the membranes isolated from fragmented nerve. Depending on the plane of sectioning, thin bands pare off from the concentrically arranged rings forming the sheath in transverse sections, or laminated slabs of tenuous ribbons are found when cutting the sheath longitudinally. This exfoliation of thin membranes from an ordered laminated structure of the sheath is best demonstrated in unembedded osmium-fixed sections of peripheral and central nerve fibres, where separation along natural cleavage boundaries can readily take place. The myelin lamellae occur single or stacked up in multiple layers (Fig. 5) where up to 10–15 unit membranes can be counted. The unit layer appears in unshadowed preparations as a transparent, flexible film of uniform thickness, with a characteristic higher density than the supporting collodion film. Even without metal shadowing their thickness can be estimated in preparations contaminated with axon filaments, by noticing that the 100 Å wide filaments fit in approximately with the edges of the unit lamellae. No surface structures are visible other than dense osmium precipitates or irregular cribriform patches in poorly fixed lamellae. But after light metal shadow-casting the myelin lamellae exhibit a characteristic surface structure consisting of closely packed granules 20–50 Å in diameter. Accurate determinations of the layer thickness can also be carried out

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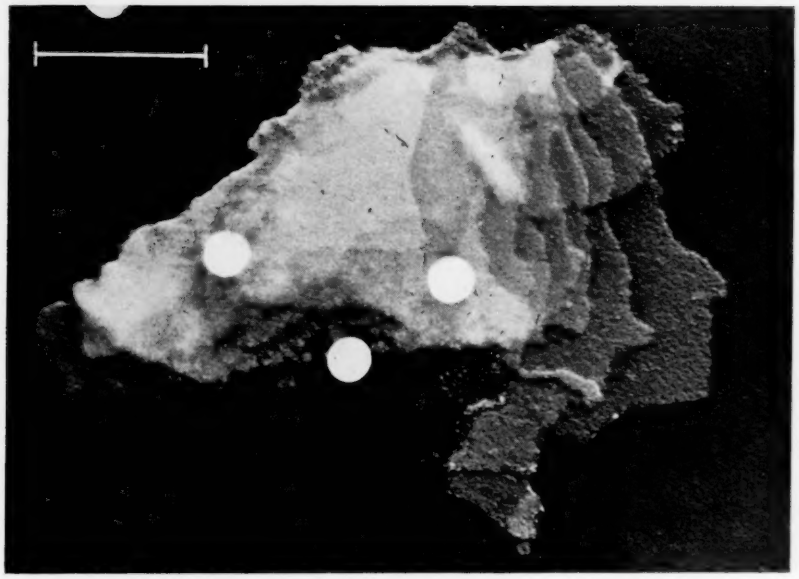

Fig. 5. Pile of 8 myelin lamellae isolated from the myelin sheath of osmium-fixed cat motor root fibres. Dow Latex calibration particles (2600 Å) have been added to determine the thickness of the shadowed lamellae

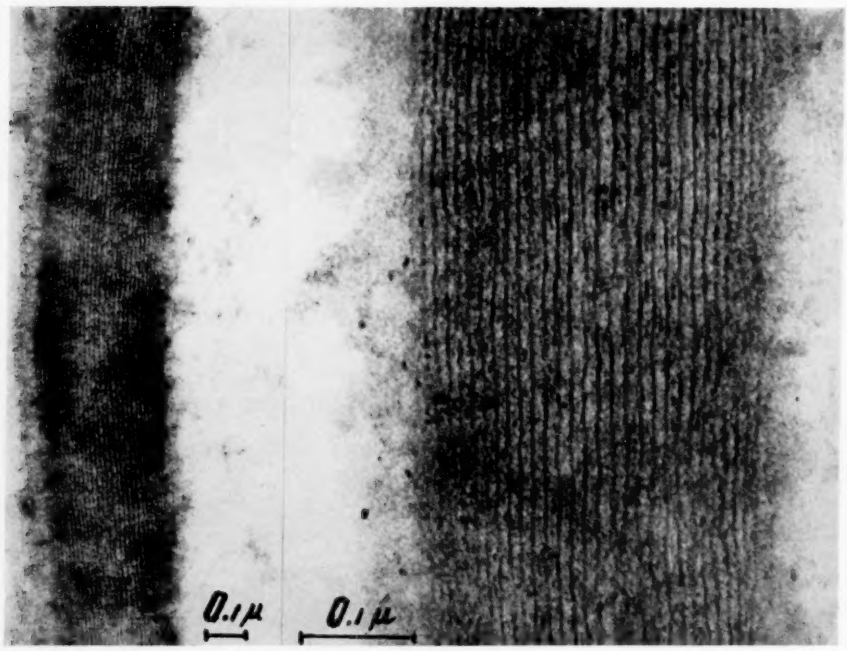

Fig. 6. Ultrathin section through the myelin sheath of osmium-fixed nerve fibre of the mouse sciatic nerve. The dark lines represent the principal period. In between these lines a faint line with dark dots is visible. The thickness of the dark lines is 25 Å. The mean distance between the principal lines is in this specimen 113 Å, the space in between these lines therefore being 85–90 Å wide. (Electron micrograph kindly provided by Dr. F. S. SJÖSTRAND)

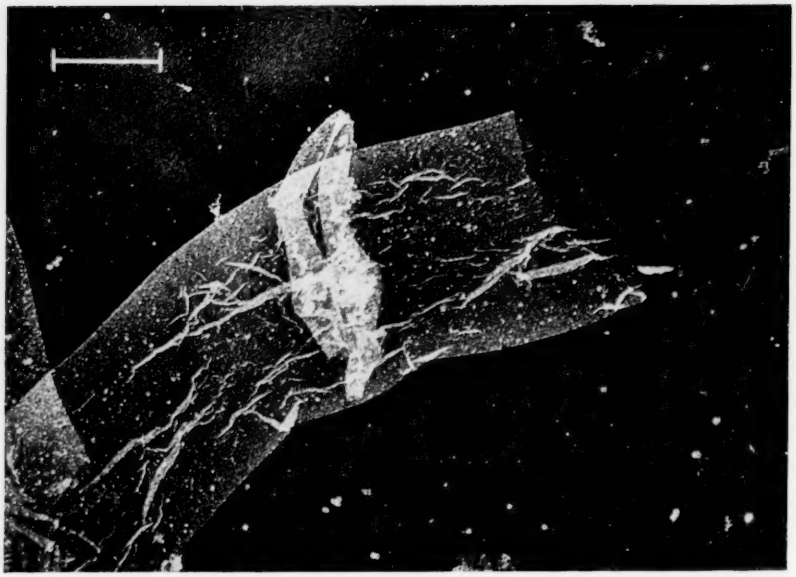

Fig. 7. Single tubular myelin membrane isolated from small osmium-fixed myelinated fibre of the frog spinal cord

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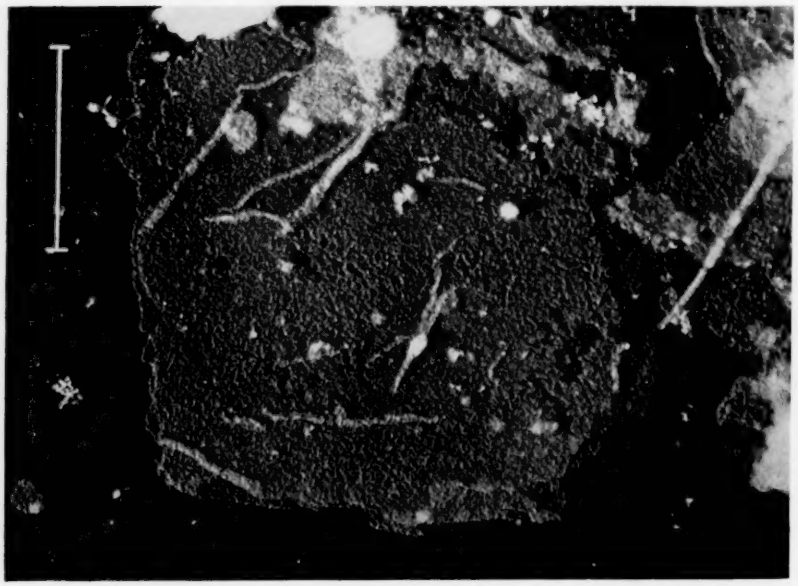

Fig. 8. Trypsin digested myelin membrane from rat sciatic nerve showing the dissociation of the unit layer into two membranes 40–50 Å thick

on these shadowed preparations by adding spherical Dow latex particles (Fig. 5) of known diameter. The average thickness of the unit lamellae in different types of peripheral and central myelinated nerve fibres was found to be 80 Å.[40], [44] The values vary of course according to the nerve type and the preparation of the specimen. The thinnest unit lamellae in air-dried preparations of frog myelin sheaths are 60–70 Å thick, and thicker ones of 80–90 Å are found in rat, cat, rabbit and human nerves. However, osmium-fixed lamellae of the rat which have been frozen-dried directly on the specimen film show a higher average thickness of 100–110 Å which probably corresponds more closely to their normal thickness in the wet state.

The granular surface of the layers is comparatively smooth with no indications of a regular array, and irregular accumulations of granules or droplets are usually fixation artefacts. In a limited number of specimens, however, the granules are aligned in a regular surface pattern within small "ordered" regions of the lamellae. Apparently well fixed lamellae often show a tendency to break up at the edges into rod-shaped granules approximately 20–50 Å wide and 50–100 Å high. Isolated lamellae piles from fragmented preparations generally appear orderly stratified in plane formations, but larger pieces can be clearly recognized as segments of a tubular structure. This is particularly evident in preparations of central nerve fibres where single tubular membranes (Fig. 7) several microns in diameter can be isolated. There are also numerous transitions between these whole, seamless tubes showing only minor creases, and thicker intact tubular formations consisting of a few membrane layers which correspond to the thinnest myelin sheaths of central nerve fibres.

The myelin membranes are best preserved by osmium fixation, since in formalin treated nerve they often have a peculiar liquefied appearance with diffuse, rounded edges and numerous surface droplets. The same type of layered structures are also seen in myelin droplets from fresh nerve which have been simply air-dried, or in thin frozen sections of the fresh myelin sheath.[44] In both cases thin membranes (60 Å) with rounded, irregular margins and filiform extensions are found piled up in multiple layers. The granular structure of the unfixed myelin layers is generally finer than in fixed material and cannot be adequately resolved with the electron microscope.

The myelin layers can also be studied in replica-adhesion preparations of freshly dissected single nerve fibres which are fixed with osmium or formalin. After stripping off the fibrous layers and the neurilemma membrane the external layers of the myelin component are exposed by this technique. The superficial myelin layers are hereby torn off and fragmented, resulting in a scaly surface structure formed by irregular flakes or lamellae which are approximately 100 Å thick.[40]

Extraction and digestion experiments

In order to determine the effects of lipid extraction on the structure of the single myelin layers, fresh whole nerves were extracted with alcohol-ether. Frozen sections of extracted nerve show that the myelin sheath has become a network of distorted concentric layers which dissociate into smooth, transparent leaflets approximately 30–40 Å thick.[40] Extensive extraction of the lipid component from the myelin lamellae has therefore nearly completely removed the granular structure and markedly reduced the layer thickness. These modifications of the lamellar structure are not so pronounced after less complete lipid extraction with acetone or chloroform, since the granular surface structure is still partially retained after this treatment. From these experiments it was tentatively assumed that extraction of the lipids could be related to the removal of the granular structure from the myelin layers. But it cannot be ascertained if the residual smooth membranes represent the protein or a stable lipoprotein component of the normal myelin layers. Repetition of the extraction experiments on thin sections of fresh nerve[44] show that the myelin sheath structure is drastically altered by all lipid solvents. Further experiments with more refined extraction procedures must therefore be carried out before any definite conclusions can be drawn from these studies.

If whole fresh nerve is subjected to prolonged trypsin digestion and subsequently fixed with osmium, characteristic modifications are produced in the structure of the myelin layers.[40] The ordinarily compact myelin lamellae show a loose cribriform or fenestrated structure after trypsin digestion, which is due to their disintegration into composite rod-like granules 50–100 Å staining intensely with osmium. When the trypsin digestion is interrupted at an earlier stage it is found that a large number of the unit lamellae have been dissociated into two thinner cribriform membranes, each of which is about half as thick (40–50 Å) as the unit layer (Fig. 8). In this case trypsin digestion has apparently not only removed some of the components which bind the particles together laterally, but also uniformly broken up the rod-shaped particles into two equal sub-units. Here again it is not possible to relate these changes induced by trypsin digestion with the composition of the myelin lamellae. However, these experiments illustrate the possibility of applying controlled digestion experiments with more specific enzymes in order to break up and selectively remove the protein or lipid components without drastic alteration of the membrane structure. Preliminary microincineration experiments[44] carried out on frozen sections of fresh nerve merely indicate that variable amounts of unidentified residual ash particles can be obtained from the myelin lamellae.

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Ultracentrifugation experiments

While there is ample collateral evidence in support of the described layered structure of the myelin sheath, the possibility of a spurious lamination produced by the preparation procedures of electron microscopy still had to be considered. Earlier ultracentrifugation experiments on fresh nerve tissue[60], [78], [7] had demonstrated that certain of its visible cell constituents could be displaced from their normal position and separated. Ultracentrifugation of fresh nerve was therefore taken up in connection with electron microscopy, in order to bring about a displacement or deformation of recognizable submicroscopic components like the layers of the myelin sheath. By thus modifying the normal structure of the sheath in a controlled fashion, it appeared possible to detect preparation effects leading to an artificial lamination.

The myelin sheath of fresh nerve fibres which have been subjected to ultracentrifugation in special plastic chambers[44] is displaced from its normal position in a characteristic way. The myelin is hereby detached from the external fibrous sheath surrounding it and sedimented towards the lower end of the tubular fibre where it folds into convoluted formations, without coalescing to become a single droplet. If the sedimented myelin is sectioned and examined with the electron microscope, it is found that the myelin membranes show multiple folding and creasing, but are otherwise unaltered. This reproducible crinkling of the layered structure can only be accounted for by assuming that the membranes were already present as regularly stratified, plane formations in the fresh myelin sheath before the warping effects of ultracentrifugation set in. The results were therefore interpreted as further indication that the myelin layers are preformed natural structures of the sheath.[44]

Crystalline structures resembling myelin lamellae

In osmium-fixed nerve certain thin lamellar structures were encountered, which exhibit typical crystal interference phenomena and can therefore be clearly distinguished from the natural lamellar membranes of the myelin sheath. These crystalline lamellae, tentatively called "osmium lamellae",[41] are thin (50–60 Å) smooth, plane flakes with angular edges which occur in piles of various thickness. They generally demonstrate crystal interference effects like "reflection images" observed close to the lamellae, or dark sinuous lines corresponding to extinction contours of the type seen also in electron micrographs of mica lamellae. Although these crystalline lamellae often closely resemble the fragments of myelin membranes in unshadowed preparations, they can be clearly recognized by recording their electron diffraction patterns. For this purpose an arrangement must be used which makes it possible to obtain

successive electron diffraction patterns and electron micrographs from the same restricted area of an object in the electron microscope.[41] With this arrangement single crystal cross-grating patterns have been recorded from the thin crystalline lamellae, and characteristic Kikuchi line patterns from the thicker piles.[44]

There are indications that the crystalline lamellae may be osmium products of some kind deposited in the sheath, since they are found closely associated with the myelin membranes only in osmium-fixed preparations. Crystalline lamellae of the same type have also been found in osmium-treated lipids extracted from nerve. But from these preliminary observations it is not possible to determine if the crystalline lamellae represent ordinary osmium reaction products, or osmium-lipid compounds formed in the nerve fibre during the process of fixation.

Crystalline structures resembling the osmium lamellae have been found recently[45] in fresh nerves subjected to the Windaus test for cholesterol by treatment with digitonine solutions. According to COLLIN and CHAVAROT[21] free cholesterol can be detected in fresh nerve fibres by applying a 0·5 per cent solution of digitonine in alcohol, which immediately produces crystals of the digitonine-cholesterol complex within the myelin sheath. Upon repeating these experiments on fresh nerves or on thin frozen sections of fresh nerve fibres, the described crystalline inclusions were found embedded in the myelin sheath. Examination of these sections with the electron microscope shows that many of the crystalline inclusions are formed by piles of thin smooth lamellae which exhibit crystal interference phenomena and are similar to the crystalline lamellae of osmium-fixed preparations. But the cholesterol-digitonine complex also forms other types of crystalline structures, and only further studies can determine if there is any relation between the lamellar crystalline inclusions and the free cholesterol present in the myelin sheath.

The concentric laminated structure of the myelin sheath

In order to determine the arrangement of the myelin layers and reveal directly the anticipated highly ordered sheath structure, it was necessary to examine adequately prepared thin sections. While studying the localization of the myelin layers, the concentric laminated structure of the sheath was first demonstrated in ultra-thin frozen sections of osmium-fixed nerve fibres by FERNÁNDEZ-MORÁN.[40], [44] In these transverse and longitudinal thin sections the sheath exhibits a perfectly regular concentric laminated structure consisting of parallel, uniform dark lines separated by lighter spaces with an average period of 80 Å. The laminated structure is seen extending uninterrupted throughout the entire myelin sheath in well-preserved sections and probably represents one of the most striking examples of extreme structural

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regularity visible over such comparatively large segments in biological objects.

The parallel dark lines marking the lamination have a thickness of 20–30 Å and appear separated by uniform lighter spaces giving a regular period, which varies according to the nerve type and the preparation of the specimen between 60 Å and 100 Å. The smallest spacings of 60–70 Å were found in frog peripheral nerves, while the largest periods of 80–100 Å have been recorded in rat, cat and human nerves. The fine structure of the dark lines cannot be clearly resolved, although there are indications of a fine segmentation of the order of 20 Å. In suitable frozen sections a faint intermediate line can also be made out between the darker main lines. Direct transitions can be seen where the laminated structure separates into thin lamellar ribbons tilted out of position to expose their broad surface. The number of lines, corresponding to the concentrically arranged myelin layers, varies from over one hundred in the thick myelin sheaths of larger nerves to a few still perfectly concentric lines in the thinnest sheaths. However, the thinnest frozen sections of unembedded nerve clearly showing the laminated structure are seldom intact over large areas, so that a complete visualization of the lamination was only possible in the smaller fibres.

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The concentric lamination is seen equally well in transverse and longitudinal sections, occupying the entire myelin sheath and constituting the only type of structure present, with the exception of irregularly distributed crevices which are probably preparation artefacts.

These observations were subsequently confirmed by SJÖSTRAND[127] who also succeeded in demonstrating the concentric lamination of the sheath in osmium-fixed guinea-pig sciatic nerves with a special ultrathin sectioning method. Periodic striations with a similar period of 50–80 Å are described in these sections of nerve embedded in collodion-paraffin, and it is assumed that they correspond to the concentric arrangement of the membranes in the sheath. Quite recently, SJÖSTRAND[128] has been able to demonstrate the concentric lamination even more clearly and with complete preservation of the sheath's structural integrity, by means of improved sectioning and methacrylate embedding techniques. In his interesting micrographs (Fig. 6) the perfectly regular concentric laminated structure is exhibited distinctly throughout the whole myelin sheath with the unmarred precision of an optical grating. The better preservation achieved in these ultrathin sections of embedded material also makes it possible to resolve the intermediate line and the details of the laminated structure with greater clarity. Measurements carried out on these embedded sections of osmium-fixed mouse sciatic nerve give values which are in good agreement with the corresponding spacings determined in frozen sections. The dark lines have a thickness of 25 Å with a 80–90 Å space in between to give a period of about 110 Å,

which is only slightly higher than the 80–100 Å period of mammalian nerves measured in frozen sections. The interperiod is described as a faint line with dark dots.

The concentric laminated structure has only been seen in osmium-fixed myelin sheaths, since the lipids in formalin fixed or ultrathin sections of fresh nerve are not well enough stabilized to exhibit this degree of fine structure. The appearance of the myelin sheath in formalin-fixed and embedded nerve which has been described as "an anastomosing lamellar net or cage"[99] is obviously due to the severe damage caused by extraction of the lipids. Dense irregular particles are frequently encountered in osmium fixed sections which show a concentric arrangement paralleling the laminated structure, and might represent oriented osmium reaction products or precipitated osmium-lipid compounds. HARTMANN[57] describes concentrically arranged placodes in the myelin sheath of osmium-fixed rat nerves which closely resemble these oriented osmium deposits. The concentric grating structure described in frozen sections[40] as a perfectly regular system of lines superimposed on the laminated sheath structure is mainly observed in areas containing these osmium deposits, and may possibly bear relation to the piles of crystalline osmium lamellae. The only discontinuities seen in the sheath are oblique clefts corresponding to the Schmidt-Lantermann incisions which extend from the axon to the external sheath layers. These clefts have also been described as conical partitions or membranes in formalin-fixed nerve.[99] The details of the sheath structure at the clefts, around the boundary region between axon and myelin sheath, and particularly at the nodes have not been studied well enough yet. The possible existence of reinforcing structures at these points remains to be determined.

From the available information on the isolated myelin layers and on the concentric laminated structure, it can therefore be concluded that the myelin sheath is built up of concentrically arranged uniform layers or membranes. This is in satisfactory agreement with the results of polarized light and x-ray diffraction studies which indicate that the myelin sheath consists of concentrically arranged lipid-protein layers. However, the average thickness of 80 Å determined for the single layers would correspond to only half the value of the 144–158 Å, x-ray spacing recorded from dried myelinated nerves. Two adjacent layers of the type revealed by electron microscopy would therefore be necessary to contribute the x-ray unit spacing. The required slight difference in geometry and composition between the layers also remains to be demonstrated, since the layers appear identical in osmium-fixed sections. The possible bearing of the faint interperiod structure on the observed alternation of intensities of the even and odd orders in the small-angle diffraction pattern of fresh nerve is likewise of interest.

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Digestion experiments indicate that each myelin layer may dissociate into two membranes 40–50 Å in thickness, which would be of the order of magnitude of the interperiod structure. In evaluating the electron microscope findings the effects of osmium fixation and the shrinkage introduced by dehydration of the specimen must be taken into account. The permeation effects of the embedding medium and possible distortions introduced by sectioning are also important. With improved preparation techniques a better preservation of the normal sheath structure can be achieved, and then a complete correlation between the results of x-ray diffraction and electron microscopy will probably be possible.

The disk layers of the retinal rods

As already deduced from polarized light studies and subsequently borne out by direct observation with the electron microscope, there is a notable similarity between the layers of the myelin sheath and the submicroscopic layers of the outer segments of the retinal rods. Polarized light analysis[122], [102], [103] indicated that the rod outer segments consist of alternate layers of lipids and proteins, and this type of layered structure found further support in their tendency to dissociate into thin platelets.[122] SJÖSTRAND[125] demonstrated these circular platelets directly, and described the details of their structure in an electron microscope study of the outer segments of guinea-pig retinal rods fragmented by sonic treatment. The outer limbs break up into thin uniform disks which are oriented with their planes perpendicular to the rod axis and have the same diameter as the rod segments. The unit disk consists of an edge-cord having a thickness of 60–90 Å, surrounding the disk membranes which are about 30 Å with dense spots of greater thickness than the disks. The edge-cord shows indications of a granular fine structure and follows the contours of the disk, being also continuous along the edges of an incision which sometimes reaches the centre of the disks. The disks show a tendency to keep associated in pairs during fragmentation and are packed with edge-cords in close contact. The dense knobs covering the entire surface of the membrane have a diameter varying between 50–250 Å and appear to be conglomerates of small granules. It is admitted however, that this aggregation of material in the dense spots may constitute preparation artefacts. Correlating these findings with the results of polarization optical analysis, the disks are considered to be mainly protein structures, but no evidence of continuous lipid layers between the disks has been found. It was tentatively assumed therefore that the lipids may be located in the areas of the dense spots.[125]

By studying ultrathin sections of embedded material, SJÖSTRAND[129] has extended these observations and shown that the outer segment of

the guinea pig retinal rods consists of about 700 double membrane disks 140 Å thick. The disks consist of two membranes 30 Å thick which are connected all around the edges and appear separated by a space of 70–80 Å (Fig. 12). The data supplied by this detailed analysis of the retinal rods establishes the essential similarity existing between the retinal disks and the myelin membranes, which seem to differ only as regards the external shape and certain dimensions of the unit structures.

Similar disk-like structures have recently been described in fragmentation preparations and frozen sections of the outer segments of frog retinal rods by FERNÁNDEZ-MORÁN.[45] These rod segments dissociate into round or oval membranes with a diameter of about 6 μ and numerous deep incisions along the edges giving them a characteristic segmented form (Fig. 9). A well-defined cord-like structure approximately 80–100 Å thick and 150–200 Å wide is continuous with the edges of the membrane all along the incisions which are of variable length, dividing the disk into irregular paddle-shaped sectors. Frequently the edge-cords show a regular structure consisting of aligned granules 100–200 Å in diameter. In the isolated single disks (Fig. 10) the membranes are approximately 70–80 Å thick and closely resemble the myelin membranes, since they appear completely smooth with no indications of the dense spots found on the surface of the guinea-pig disks. The membranes exhibit a very fine granular surface structure, but instead of breaking down into granules they show a tendency to disintegrate into peculiar contorted threads reminiscent of submicroscopic myelin figures. In some preparations the membranes of the single disks appear to consist of two membranes about 30–40 Å thick which are pressed together upon drying. The disks are usually found stacked up in piles where their number can be determined by counting the closely packed edge-cords (Fig. 11). Assuming an arrangement of the disks similar to that of the guinea pig rods, each outer segment of the frog retinal rods which is 40–50 μ long would contain about 2000–2500 disks.

The Neurilemma and Schwann cell

In electron microscope preparations the thin transparent neurilemma of light microscopy can be recognized as a well-defined membranous structure consisting of a fine granular membrane with reinforcing bundles of submicroscopic fibrils exhibiting typical collagen periodicity (Fig. 13). Were they to exist as separate structures, neither the isolated membrane which is barely a few hundred Å thick nor the network of submicroscopic fibrils could be resolved with the light microscope. Their intimate association, however, and the accumulation of evenly distributed fibril bundles renders the whole visible as the tenuous membrane of Schwann or neurilemma.

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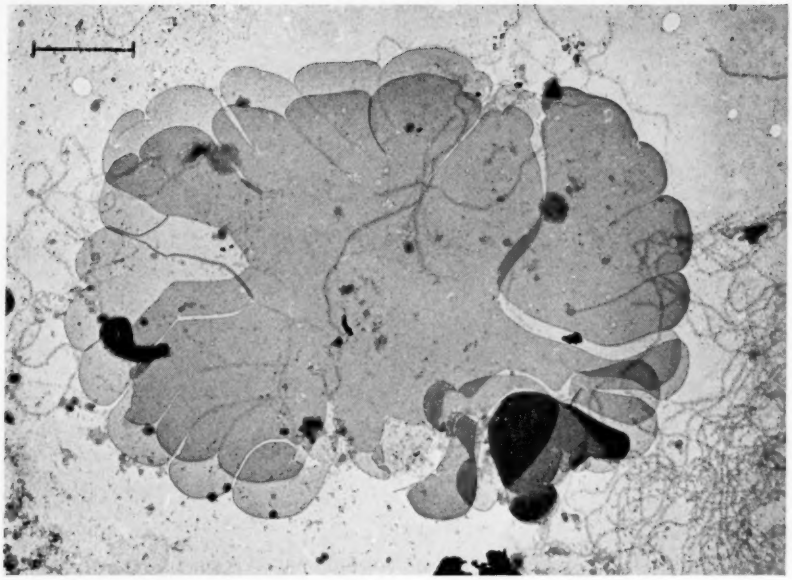

Fig. 9. Two unit disks isolated from the outer segments of frog retinal rods, and associated fibrils with characteristic cross striations. Osmium-fixed, unshadowed preparation

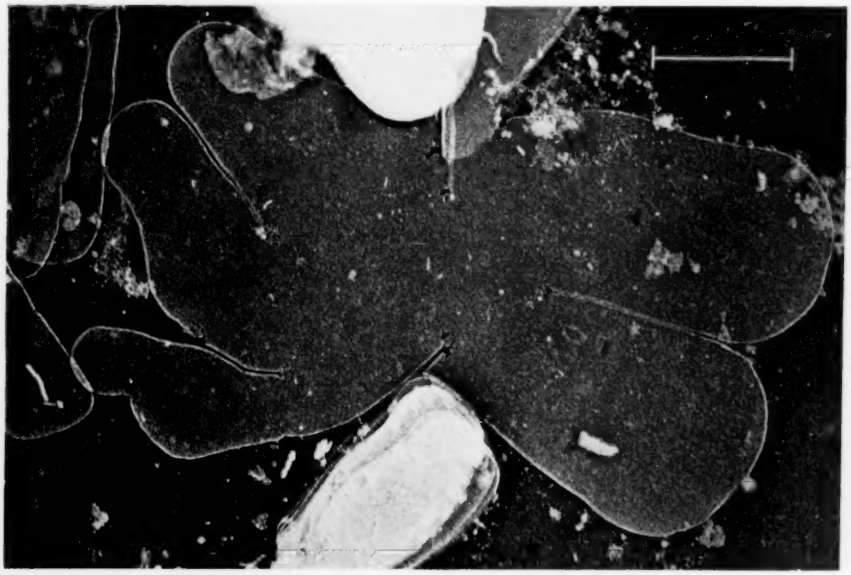

Fig. 10. Single unit disk isolated from the outer segment of frog retinal rods, showing the smooth membrane with granular surface structure and the edge-cord. Osmium-fixed, platinum-shadowed preparation

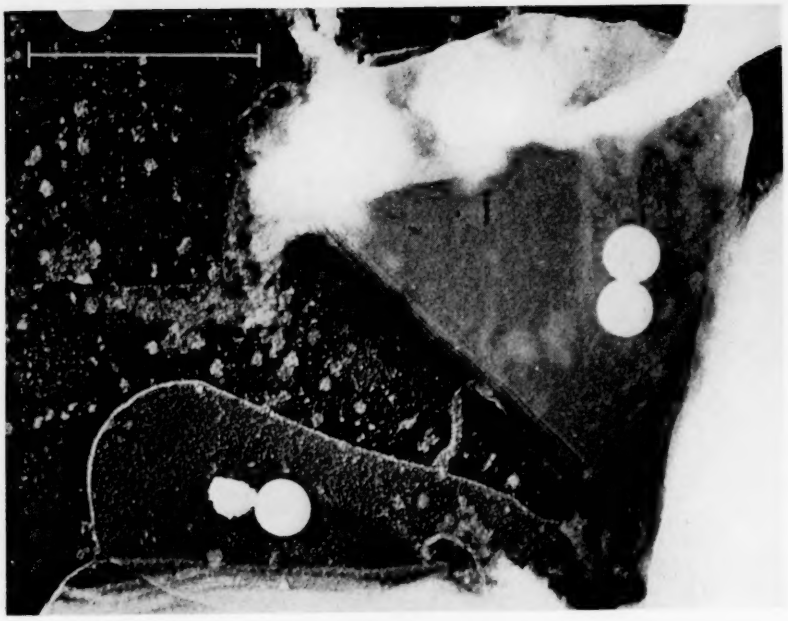

Fig. 11. Pile of 6 unit disk segments isolated from the outer segment of frog retinal rods, showing the edge-cords in close contact. Dow Latex calibration particles have been added

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Fig. 12. Ultrathin longitudinal section through the basal part of the outer segment of a retinal rod from guinea pig eye. The outer segment is composed of double membrane disks, the mean total thickness of which is 140 Å. The constituent two membranes measure 30 Å in thickness, and the width of the space in between these membranes is 70–80 Å. The distance between double membrane disks varies between 100–200 Å due to differences regarding the preparation technique. (Electron micrograph kindly provided by Dr. F. S. SJÖSTRAND)

The neurilemma membrane with its fibrillar reinforcements forms a continuous tubular envelope around the myelin sheath in the internode portion, as shown by a study of serial transverse and longitudinal thin sections. In transverse frozen sections the intact fibrous membrane often separates completely from the myelin sheath as a tubular band. This is in agreement with the results of micro-manipulation experiments[23] and studies on living nerve[130] which indicate that the neurilemma is a separate structure. The membrane itself has an average thickness of 200 Å, although thicker membranes (up to 400 Å) have been observed in large myelinated mammalian fibres. It shows a uniform fine granular structure which is removed upon extraction with lipid solvents, leaving a thin fenestrated membrane (approximately 50 Å) with faint residual granules.[40], [44]

Although resembling the myelin membranes in this respect, the neurilemma membrane can be distinguished from the myelin layers because of its greater thickness and close association with the fibrous network. The most common type of fibrils found adhering to the membrane are 500–700 Å wide, of indefinite length, with an axial repeating period of 600–660 Å and the same type of intraperiod structure which is characteristic of collagen fibres.[52], [53] The fibrils generally associate to form bundles of varying thickness with their axial periodicity in nearly perfect register. Predominantly longitudinally oriented fibril bundles interlace with circular fibres to form a fairly dense network distributed uniformly over the entire membrane. The fibrous net is so firmly attached to the granular membrane that it must contribute decisively to the mechanical stability of the neurilemma.[121] This fibrous network would therefore correspond to the "inner endoneurium" or sheath of PLENK and LAIDLAW[89] described as a web of very fine argyrophil fibres. Preliminary observations on silver stained nerve fibres show that the colloidal silver particles precipitate on the surface of the neurilemma fibrils in a pattern characteristic of such argyrophile reticulin fibres.[58] LEPINE and CROISSANT[70] likewise describe fibres with collagen periodicity covering the myelin, and state that this "neurocollagen" is not attacked by the collagenase extracted from microbial toxines. These fibres forming an integral part of the neurilemma membrane can be distinguished from the outer endoneurium or sheath of KEY and RETZIUS[63] which consists of more compact bundles of collagenous fibrils.

In addition to the fibres with collagen periodicity there is another type of "smooth" fibrils 100–200 Å in diameter generally bound together in compact longitudinal strands.[40] Since these dense smooth fibres are resistant to pepsin digestion and potassium hydroxide solutions they may bear relation to the elastic tissue elements postulated in Henle's sheath.[23]

Rozsa, Morgan, Szent-Györgyi and Wyckoff[99] describe the neurilemma as a fibrous membrane, approximately 500 Å thick, which "seemingly is a close net of very delicate fibres", in sciatic nerve fibres of the rabbit. Since the nerves were formalin fixed and embedded in methacrylate after alcohol or pyridine dehydration, only the fibrous components of the neurilemma are well preserved in these preparations. According to these authors the neurilemma is especially noticeable at the Ranvier nodes where it dips inwards to the constricted axon, and its internal threads are particularly well oriented and closely packed. The fibrous layers binding the nerve fibres together are considered to be bundles of connective tissue filaments with the same characteristic cross striations as collagen from other tissues. In well-preserved osmium-fixed sections of rat nerves Pease and Baker[87] describe the neurilemma as a distinct sheath present over the whole nerve fibre and at least partly made up of collagenous fibrils, with an axial periodicity of 640 Å.

The relation of the Schwann cell to the neurilemma is difficult to determine because only the condensation of cytoplasm around the Schwann nucleus can be made out clearly in sectioned material. In many preparations the cytoplasm of the Schwann cell seems to be continuous with the granular neurilemma membrane, but in others the Schwann cell nuclei appear interposed between the neurilemma and the myelin sheath.[87] This question demands further investigation, particularly as regards the claims based on observations with the light microscope that the protoplasm of the Schwann cell behaves differently from the neurilemma membrane in degenerating nerve.[137]

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The Schwann cell

In sections through the Schwann cell nucleus large dense bodies resembling nucleoli are found embedded in a fine granular material. The nucleus is surrounded by granular cytoplasmic masses and numerous corpuscles of different sizes. Most characteristic among these are certain dense oval bodies 0·3–1 μ in diameter containing a finely granular material which can be extracted with alcohol or acetone.[40], [44] They stain intensely with osmium, seem to be bounded by a membrane and probably correspond to the Reich granula.[94] Elongated bodies resembling mitochondria with a very fine laminated internal structure are also present in the Schwann cell cytoplasm. Investigation of the Schwann cell is still in a preliminary stage, and much remains to be done by combining cytochemical methods with electron microscopy.

Axon Structures

Electron microscope studies of the axon's structure have been pursued with special interest in view of the characteristic neurofibrils revealed

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Fig. 13. Neurilemma membrane with longitudinally oriented bundles of fibrils exhibiting collagen periodicity, from a longitudinal frozen section through rat sciatic nerve

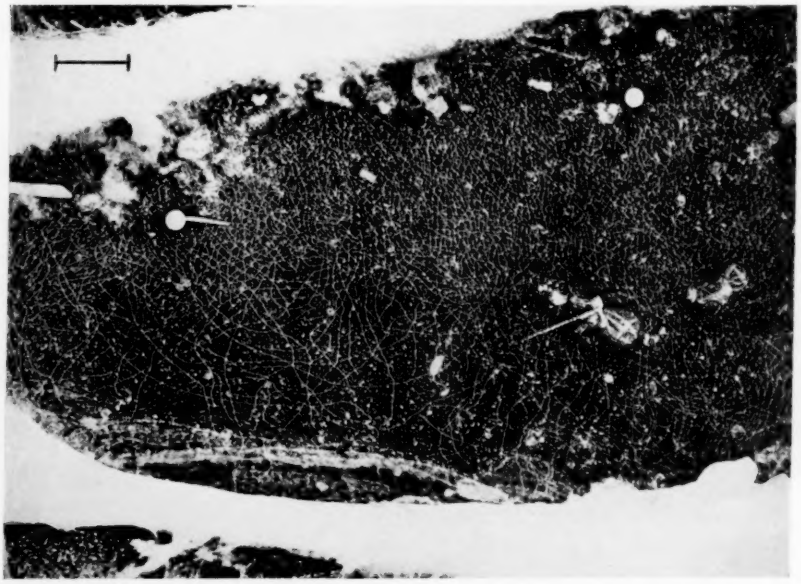

Fig. 14. Longitudinal section through the axon of a frog sciatic nerve fibre showing the axon filaments which have an average width of 100 Å. Fresh frozen section fixed with osmium vapours

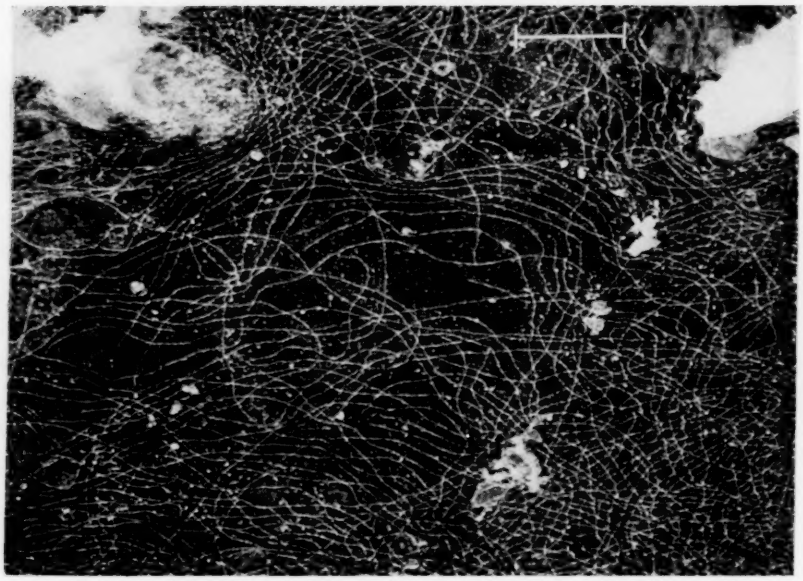

Fig. 15. Axon segment from a longitudinal frozen section of fresh nerve subsequently fixed with osmium. Notice parallel array of the longitudinally oriented axon filaments

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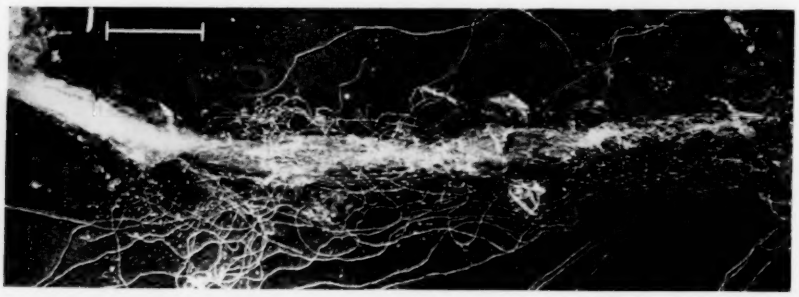

Fig. 16*a*. Fine unmyelinated nerve fibre from the lateral funiculus of frog spinal cord. The fibre is partly invested with a thin granular sheath and separates into bundles of thin (200 Å) axon filaments

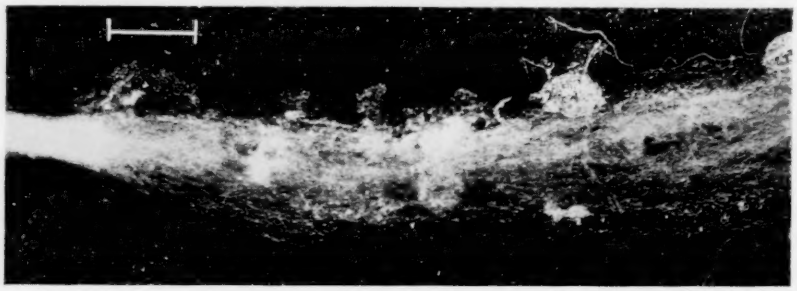

Fig. 16*b*. Unmyelinated nerve fibre from the lateral funiculus of rat spinal cord. The compact axon dissociates into numerous thin nodose filaments (100–200 Å). Remnants of the granular sheath and its oval bodies are seen.

by histological procedures, and the results of polarized light analysis indicating the presence of oriented elongate particles in the axon. RICHARDS, ANDERSON and STEINBACH[98] examined extruded squid axoplasm and found contorted fibrillar structures composed of linearly aggregated elongate particles about 150 × 500 Å in size. Similar contorted filaments have also been observed in unfixed material sucked up from the axon of giant nerve fibres of the earthworm.[44] DE ROBERTIS and SCHMITT[25], [26] described fibrils approximately 500–1000 Å in width, with dense edges, low density in the central axis and periodic cross striations in formalin-fixed nerves subjected to sonic fragmentation. These fibrils were tentatively called "neurotubules" and considered to be axonic constituents. Subsequent investigations have indicated, however, that these dense-edged fibrils are most probably collagen fibrils of the external sheath layers which present this spurious tubular appearance due to contamination with an undetermined diffuse substance.[40] Upon reinvestigating the origin of the "neurotubules" SCHMITT[109] and SCHMITT and GEREN[118] also concluded that the dense-edged fibrils are not axonic constituents but probably derive from the sheath. In replica-adhesion preparations of isolated nerve fibres FERNÁNDEZ-MORÁN[38] observed a network of fibrils approximately 100 Å in diameter with an axial periodicity of 200 Å surrounding the axis cylinder. The fibrous constituents of formalin-fixed and unfixed axoplasm of giant fibres of the squid and the marine annelid Myxicola were examined in fragmented preparations by SCHMITT.[109], [111] It was shown that the fixed axon contains nodose filaments of indefinite length and having widths ranging between 75 and 200 Å, while in unfixed material filaments 100–150 Å wide, with relatively smooth edges were found.

With the development of suitable sectioning techniques it became possible to localize the described filaments and study their arrangement within the axon. In frozen sections of osmium-fixed frog and rat nerves FERNÁNDEZ-MORÁN[39], [40] described long nodose filaments with a diameter of 100–200 Å running in an oblique or longitudinal direction to form a network within the axon. A dense net of interlaced axon filaments coating the internal sheath wall was also regularly seen, and it was therefore assumed that it corresponded to the axolemma membrane.

SCHMITT and GEREN[118] examined thin sections of osmium- and formalin-fixed squid, frog, rat and human nerves embedded in methacrylate, and found the same type of axon filaments having diameters ranging from 100 to 200 Å and indefinite length. These filaments frequently have a nodose appearance due to axial discontinuities spaced fairly regularly at intervals of 200–350 Å. Similar filaments have been subsequently described in thin sections through the axon

of osmium-fixed, embedded nerve by BAUD,[5] BRETSCHNEIDER,[18] DUNCAN and ANTES[30] and other investigators.[87] ROZSA, MORGAN, SZENT-GYÖRGYI and WYCKOFF[99] also demonstrated a filamentous structure of these dimensions running parallel to the axis of the axon or forming a delicate network in formalin-fixed rabbit nerves, and in their micrographs there is no evidence of an axolemma membrane.

These authors[99] and PEASE and BAKER[87] have made the important observation that the axon is only constricted, but not interrupted at the nodes of Ranvier, with no evidence of a transverse membrane. In addition to these fibrous structures, dense rod-shaped particles of different widths and up to several microns long which are referred to as filamentous mitochondria, have been described within the axon.[40], [118], [99], [87] Dense oval corpuscles and clear vesicular bodies are also regularly found in frozen sections through the axon of fresh nerve fibres.[44] Longitudinally oriented, smooth axon filaments with a diameter of 100–200 Å can also be demonstrated in fresh nerve sections which have been frozen dried directly or simply air-dried without fixing.[44] The fibrous axon structures are especially well preserved in comparatively thick (1–2 μ) frozen sections of fresh nerve which are fixed with osmium vapour (Figs. 14, 15). Whereas in ultrathin sections of embedded nerve the filaments are found severed into short segments, the use of these thick frozen sections devoid of an embedding medium makes it possible to observe the unbroken longitudinal course of the individual filaments over long stretches of 50 μ or more. In well prepared sections the filaments are orientated parallel to the fibre axis in orderly array (Fig. 15). The axon filaments can aggregate to form compact bundles which become visible as neurofibrils in sections of fresh nerve under the action of osmium fixation. In these sections of fresh nerve as in all other preparations the axon filaments invariably appear "solid" with clearly defined contours, and double-edged structures of the "neurotubule" type are not encountered.

Recently DE ROBERTIS and SOTELO[28] have studied cultured nerve tissue from chick embryos with the electron microscope and described long cylindrical fibrous elements in the more differentiated cells. These dense-edged elements have a diameter of 300–500 Å and are also called neurotubules because of their similarity with the dense-edged fibrils of fragmented preparations. The cytoplasm contains isolated micro-vesicles 300 to 500 Å in diameter, which show a tendency to fuse into these double-edged cylindrical structures. Although it is possible that such neurotubular structures may appear under the special conditions present in tissue cultures, there is as yet no evidence that they are axon components of normal nerve. The ease with which "neurotubules" of any dimension can be produced by lipid contamination of ordinary fibrous structures, and the subsequent abolishment of the tubular

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appearance by addition of alcohol or acetone weighs heavily against their existing as normal constituents of nerve. Ultracentrifugation experiments performed on whole fresh nerve[44] produce stratified meniscus formations within the axon which are due to displacement and sedimentation of the axon filaments in these regions. This displacement and concentration of filaments, which still retain their characteristic structure under the effects of ultracentrifugations, would indicate that they are natural preformed elements in the fresh axon. This is in accord with earlier ultracentrifugation experiments performed on spinal ganglion cells,[60], [78], [7] which also showed that the neurofibrils may be displaced and concentrated. Digestion with pepsin and trypsin destroys the axon filaments, but they are resistant to extraction with alcohol and other lipid solvents.[44]

Microincineration experiments performed on thin frozen sections of fresh nerve[44] reveal a comparatively large amount of mineral residue in the axon, part of which is aligned in rows of minute granules suggesting the residual ash of axon filaments. The dense net of interlaced filaments previously described as the axolemma membrane in osmium fixed whole nerve could not be seen in fresh sections.[44] Instead it was found that the axon filaments were more concentrated around the internal sheath wall where they associate with the sheath membranes to form a distinct boundary layer.

All the available evidence points therefore to the existence of these longitudinally oriented axon filaments having widths of 100 Å and indefinite length as normal constituents of fresh axoplasm. Referring to PETERFI's[88] suggestion that the fresh axon is a rodlet sol capable of forming fibrous structures under slight chemical provocation, it is conceivable that the filaments are produced by linear aggregation of elongate colloidal particles.[109] But our knowledge of the axon filaments is barely incipient, and there is yet no way of determining the conditions under which they normally exist in the living axon and the modifications they may undergo during different states of activity. In particular it would be of interest to determine the relation of the axon filaments to the flow of axoplasm[135], [138] and the viscosity changes of the axon in living nerve.

No data are available on the chemical composition of the axon filaments. It has been suggested[109] that the squid axon filaments may be related to the protein complex "neuronin" isolated from squid axoplasm by BEAR, SCHMITT and YOUNG.[12] These investigations already indicated that the normal state of aggregation of the axon proteins is very sensitive to the effects of ionic strength, *p*H and specific ions. SCHMITT *et al.*[108], [109], [111] are making systematic studies of the physical chemical properties of the squid axon proteins and have succeeded in isolating and characterizing several protein fractions.

Interesting results are to be expected from these studies, for it is evident that detailed knowledge of the physical chemical properties of axon proteins is essential for an understanding of the nerve axon's organization and function.

The Unmyelinated Nerve Fibres

Unmyelinated nerve fibres are characterized by being devoid of a clearly discernible myelin sheath when studied with ordinary histological methods. Following REMAK's classical description[95] the fibres which bear his name are thin dense bands of varying calibre with indications of a longitudinal striation, adhering long slender nuclei and an enveloping thin neurilemma membrane. Polarized light studies[29], [55], [66] suggest, however, that even the thinnest of these fibres are invested with a tenuous myelin sheath. The Remak fibres are particularly numerous in the sympathetic nervous system, where they generally present a net-like, interlacing arrangement, and in many cerebrospinal nerves. The other type of unmyelinated nerve fibres, called naked axons, are apparently devoid of all covering and occur in large numbers in the grey matter of the brain and spinal cord.[92] With appropriate staining methods closely packed neurofibrils and a perifibrillar substance can be demonstrated in the axon of unmyelinated fibres.

According to x-ray absorption studies of ENGSTRÖM and LÜTHY[35] the axon of unmyelinated fibres seems to have a greater mass per unit volume than the axon of myelinated fibres. SCHMITT and GEREN[118] examined unmyelinated nerve fibres in sections of human sympathetic nerve and described a network of nodose filaments with average widths of 200 Å in the axon, which is bounded by a thin sheath. BAUD[6] also studied unmyelinated fibres in sciatic nerves of Axolotl with the electron microscope and found that their axon was constituted by a compact net of protofibrils with reduced interfibrillary spaces.

Unmyelinated fibres from rat and frog spinal cords and Remak fibres from sympathetic trunks have been studied in thin sections and dissociation preparations by the present author.[44] These fibres are especially abundant in the white matter of the rat spinal cord, where the finest ones are barely visible and often thin enough for direct examination with the electron microscope. The unmyelinated fibres appear here as dense cylindrical cords, 0·5–2 μ in diameter, which consist of tightly packed filaments of indefinite length and with an average width of 100 Å (Fig. 16*a*, *b*). Most of the filaments have a typical nodose appearance in osmium-fixed preparations, suggesting an axial periodicity of 200–300 Å. The filaments are arranged parallel to the fibre axis and run perfectly independent, straight courses without branching or fusion despite the tight packing. There seems to be a diffuse, granular

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substance binding the filaments together, although the extremely compact appearance of the fibres is partially due to the effects of drying in these preparations. Regardless of size, all of the fibres show a thin sheath which consists of a single granular membrane approximately 60–100 Å thick. These tubular membranes are closely wrapped around the axon and show the common type of granular fine structure consisting of tightly packed rod-like granules 60–100 Å high and about 20–50 Å wide. At regular intervals, long opaque bodies resembling nuclei are attached to the membrane and appear surrounded by irregular masses of a finely granular cytoplasm. The finest unmyelinated fibres, 0·2–0·4 μ in diameter, consist of only a few axon filaments enveloped by a tubular membrane and represent transition forms to the typical submicroscopic fibres (Fig. 18). The Remak fibres of sympathetic nerves are held together by dense fascicules of fibres exhibiting a typical collagen periodicity.

These findings indicate that the so-called unmyelinated nerve fibres have the same basic submicroscopic structure as the myelinated fibres. The preponderance of axon components at the expense of the myelin sheath, which is reduced to a single membrane, seems to be the distinguishing feature of this type of fibre. In this respect, the "unmyelinated" fibres are ideal objects for the study of axon structure since the axon filaments can be examined directly with a minimum of preparation manipulations. Moreover, the perfectly regular, independent course pursued by the individual filaments throughout the entire length of the fibre affords further evidence that they are not artificially precipitated fibrous structures.

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The Submicroscopic Nerve Fibres

Since the smallest myelinated nerve fibres were found to differ from the larger fibres mainly as regards the number of the common constituent structures, the possibility was considered that even finer fibres might exist consisting of nothing more than single submicroscopic elements. While searching for these simplest forms of central nerve fibres new types of submicroscopic fibres were found, which are thin enough to display their entire structure in a form suitable for electron microscopy.[44] In sections and dissociation preparations chiefly from the lateral funiculus of the frog spinal cord these fibres appear as thin ribbons with diameters ranging between 0·1 and 1 μ and reaching lengths up to 50–100 μ. Despite their considerable length, all but the thicker ones are invisible with phase contrast, and the majority of these fibres can only be detected with the electron microscope. These elements were identified as nerve fibres because of their fine structure and the connection with larger myelinated fibres from which they arise as fine collaterals (Fig. 20*a*, *b*). Essentially, each fibre consists of a thin

sheath formed by a single tubular membrane which contains long filaments 100–200 Å in diameter (Figs. 18, 19).

In air-dried preparations the thin sheath resembles a collapsed tube fitting loosely around the few internal filaments, indicating a high water content or loss of some of the internal components in the process of drying. However, in specimens prepared by a special freezing-drying method[44] large cylindrical segments appearing "solid" alternate with thin flattened parts. When the fibres can be followed up to their point of origin, it is seen that they are attached to the myelin sheath of larger fibres (Fig. 20*a*) by means of a characteristic hillock or short cylindrical segment. According to their width and configuration the fibres can be divided into those of medium size with a diameter of 0·5–1 μ, and finer ones 0·1–0·5 μ in diameter. The fibres of medium size consist of a tubular sheath formed by a single membrane which is 60–80 Å thick, and a variable number (10–20) of fine axon filaments (Fig. 18).

The membrane closely resembles the myelin membranes of larger nerve fibres and consists of rod-like granules which are packed together tightly without the apparent contribution of a separate basal layer (Fig. 19*a*). Although the fusiform enlargements which are spaced at regular intervals along the fibre may be artificial formations, these swellings of the sheath mark the location of opaque oval bodies, 1–0·5 μ long, which are attached to the membrane. These bodies are surrounded by tiny vesicular particles, 600–2000 Å in diameter, and frequently seem to be connected by fine varicose bands adhering to the membrane. The long filaments contained within the sheath have an average diameter of 100 Å and exhibit a regular nodose axial structure, as if they were formed by linear aggregation of minute segments approximately 200 Å long. They are morphologically identical with the fine axon filaments of ordinary nerve fibres.

The finest submicroscopic fibres which are barely 600 Å wide and often over 50 μ long have reached the simplest type of organization, since they consist of a single tubular membrane containing only one central filament (Fig. 19*b*). These fibres are connected directly with the small myelinated fibres of the spinal cord or with similar fibres of medium size, and frequently show terminal formations consisting of circumscribed oval bodies about 1 μ in diameter.[44] De Robertis and Sotelo[28] have found nerve fibres growing in tissue culture which are also of submicroscopic dimensions and closely resemble these central submicroscopic fibres. However, "neurotubules" of the type described in the tissue culture preparations were not seen in these spinal cord fibres, where the axon filaments invariably appear as clear-cut, "solid" fibrils. While most of the findings indicate that the submicroscopic fibres are special types of nerve fibres, their possible relation to dendrites or to neuroglia fibrils attached to the myelin sheath must also be

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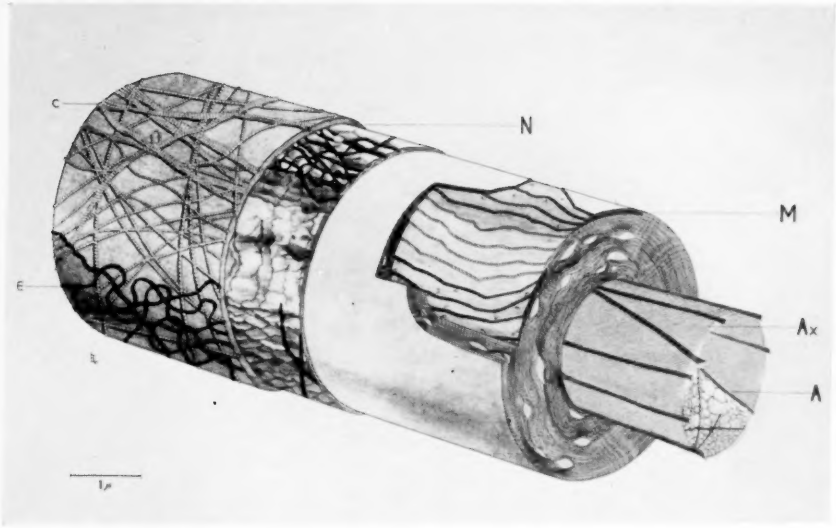


Fig. 17. Schematic diagram of the internodal portion (corresponding to a cylindroconic segment) of a myelinated nerve fibre to illustrate the general features of its submicroscopic organization. The neurilemma membrane (*N*) with attached collagen fibril bundles (*C*) and smooth fibres (*E*) envelopes the myelin sheath (*M*). The concentric laminated structure of the sheath is represented as a series of 50 thin-walled tubes fitted tightly into each other. The axolemma membrane (*Ax*) as shown here is largely a preparation artefact. The axon filaments run predominantly in a longitudinal direction within the axon (*A*)

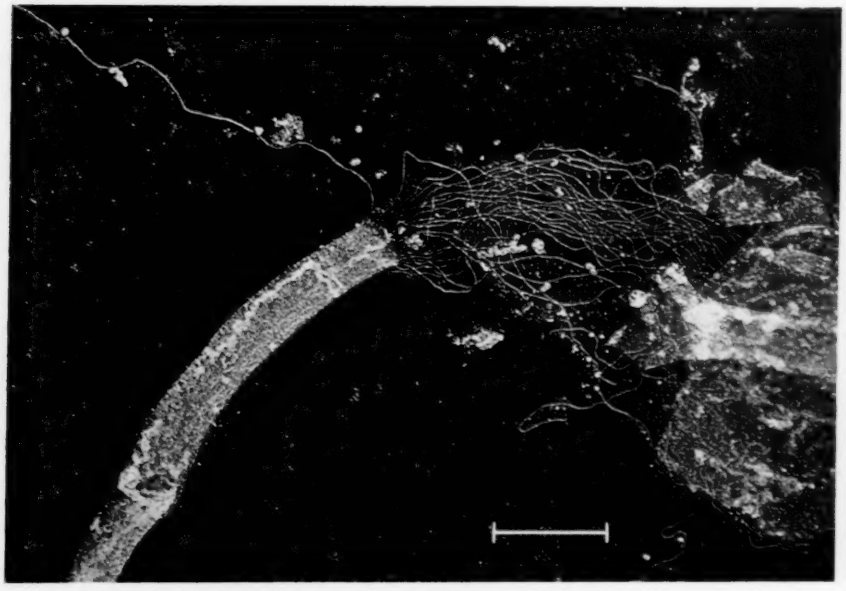


Fig. 18. Submicroscopic fibre of medium size from lateral funiculus of frog spinal cord. A bundle of 22 axon filaments (100 Å width) emerges from the broken end of the single membrane tubular sheath

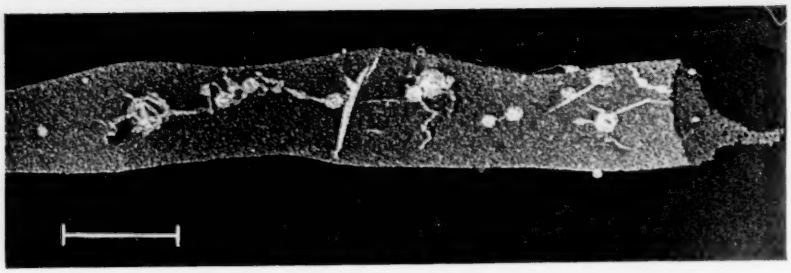


Fig. 19*a*. Single membrane sheath of a submicroscopic fibre showing its tubular character and granular structure at the broken end

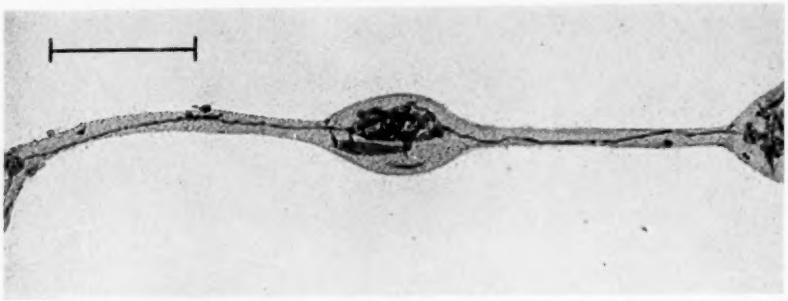
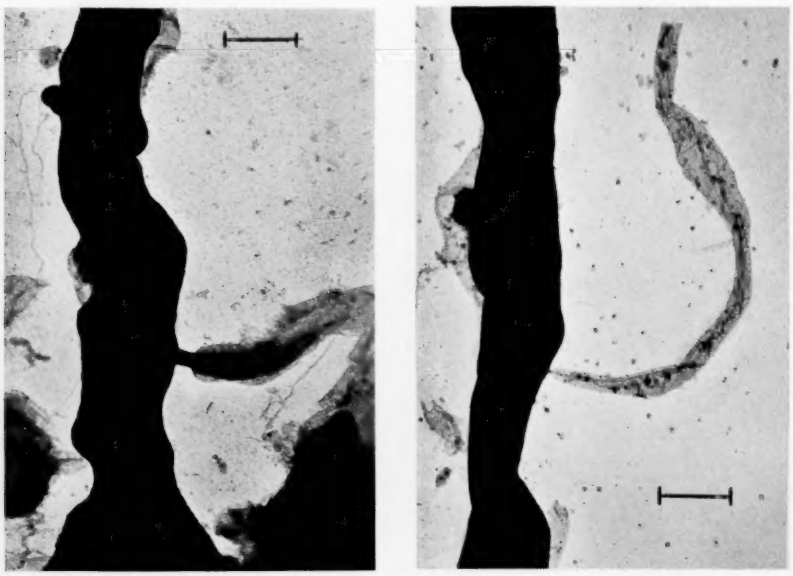


Fig. 19*b*. Fine submicroscopic nerve fibre with fusiform enlargements from the lateral funiculus of frog spinal cord. The shaft of this fibre has a width of 1000 Å and consists of a single tubular membrane fitting closely around an individual axon filament 200 Å in diameter



a *b*

Fig. 20*a*, *b*. Submicroscopic fibres emerging as fine collaterals from small myelinated nerve fibres of the frog spinal cord. A dense hillock is seen where the thin fibres arise from the myelinated nerve fibres

considered. According to BAIRATI[3], [4] the fibrous glia structures are composed of elementary fibrils presenting a periodic structure similar to that of collagen, and the glia fibrils are nearly always endocellular.

It seems unlikely that the described submicroscopic fibres are neuroglia structures, but until more is known about the structural elements that build up nerve tissues no definite conclusions can be reached. The possible identification of some of these fibres as dendrites or telodendria would not affect their fundamental structural similarity with myelinated nerve fibres. From a morphological point of view these extremely fine fibres can therefore be regarded as submicroscopic prototypes of the ordinary myelinated nerve fibres.

THE GENERAL PATTERN OF NERVE FIBRE ORGANIZATION

The results of the electron microscope studies described here may be summarized by referring to a schematic representation of nerve fibre structure which was presented at an earlier stage of these investigations.[40] Additional information obtained with improved preparation techniques has since then become available which substantiates the main features of this diagram with certain modifications. The diagram gives an integrated picture of the observed structural relations in that part of the internode portion of a myelinated nerve fibre which corresponds to a cylindroconic segment containing all of the fundamental components. It can therefore be regarded as representative of the general pattern of myelinated fibre organization, leaving out the Schwann cell, the incisions and the important node structures which have not been studied well enough yet.

Instead of the familiar image of the living nerve fibre with its opaline myelin sheath and viscous fluid axon, the diagram essentially depicts a concentrically laminated tube with a cylindrical fibrous core and an external fibrous membrane (Fig. 17). The concentric laminated structure as shown here is based primarily on its direct visualization in thin sections, which confers a striking reality to the highly ordered structure of the myelin sheath already deduced from polarized light and x-ray diffraction studies. It represents the concentric arrangement of the myelin membranes isolated by different procedures, and is also sketched in the fragmented, imbricated form revealed by the replica-adhesion technique. The arrangement of thin-walled tubes fitted tightly into each other derives from observations on thinner sheaths where single tubular membranes can be discerned, and also from the clear-cut disposition of the concentric layers in sections. If allowance is made for the modifying effects of the preparation techniques, the thickness determinations for the single layers are in agreement with the results of x-ray diffraction. There is indirect evidence from the extraction and digestion experiments that the single myelin layers are composed of

radially oriented rod-like particles, presumably lipids, which are compactly bound together by a protein framework of some kind. This would likewise be in general accord with the results of polarization optical and x-ray diffraction analysis. However, no visible structural regularity of the concentric lamination in the radial direction has been revealed by our present techniques. Whether there is also an ordered arrangement of the lipid molecules within the single layers has yet to be determined. Further investigations on the arrangement of these multiple membranes in the living fibre, and the changes that may occur during different phases of activity will require improved preparation techniques applied in combination with indirect methods of ultrastructural analysis.

The myelin membranes are essentially similar to the unit disks of the retinal rod outer segments, as shown by polarized light and electron microscope studies. It is also interesting that chloroplasts have a similar lamellar structure[49], [50] consisting of lamellae which are about 70 Å thick as clearly shown by STEINMANN.[132] The structural similarity existing between such specialized energy converting system as chloroplasts or retinal rods and the myelin sheath may be significant and possibly reflect an underlying basic functional analogy. The myelin layers also resemble the membranes of many cells[100] and the system of double membranes described in certain mitochondria.[129]

The crevices suggested in the sheath are probably preparation artefacts, since well-preserved sections generally show a uniform, uninterrupted laminated structure throughout the whole myelin sheath. However, the existence of faults or crevices in the sheath must be considered, particularly in regions adjacent to the clefts. The axon structures have only been sketched in the diagram and correspond to preliminary findings which have since been partially amended. The axolemma membrane thus represents the artificial accentuation of a greater concentration of axon filaments which is regularly found at the sheath axon boundaries. The axon is occupied by the longitudinally oriented axon filaments 100–200 Å in diameter and of indefinite length which have been described in detail. Demonstration of these fibrous elements has confirmed the conceptions of axon structure derived from polarization optical analysis and opened up a new field of investigation. In these studies, however, electron microscopy will probably be ancillary to the more important chemical and physical-chemical investigations of the axon proteins and other axon constituents.

Upon comparing the fundamentally similar sheath membrane and axon filament structures of myelinated fibres with those of unmyelinated fibres (Fig. 16) and of the submicroscopic fibres (Figs. 18, 19), a common pattern of submicroscopic organization becomes apparent. In its simplest form this pattern is represented by the association of only

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one filament with a single tubular membrane which is found in the finest submicroscopic fibres. From there on, progressive addition of further elementary membranes and filaments conforming to the same basic plan builds up all transition forms from submicroscopic fibres to the visible myelinated nerve fibres. In disclosing that these submicroscopic components are the basic structures of nerve fibres, electron microscopy presents interesting possibilities to the neurophysiologist by suggesting the existence of functional units of these dimensions. Thus the activity of a single membrane-filament system could be detected by recording directly from the simplest type of submicroscopic fibres with suitable micro-electrodes.

Now that suitable preparation techniques can be applied, the available knowledge of nerve fibre structure will serve as a basis for further studies on the degeneration and regeneration of nerve, and on the embryological development of the fibres. These studies may reveal, for example, how the myelin layers are formed and broken down during the processes of myelination and demyelination.[131] From this structural analysis at the submicroscopic level a better understanding will be gained of the fundamental processes operating during growth and regeneration of nerve fibres. Electron microscope studies of virus-infected nerve [27], [62], [97] will likewise yield important information on the properties of its submicroscopic constituents, now that more precise localization of the virus particles is possible.

In assessing the results of electron microscopy, we must keep in mind that what has been revealed so far is hardly more than the osmium-stabilized "skeleton" structures of the lipids and proteins which constitute the basis of the extremely complex chemical organization of nerve. Not until the gaps in our knowledge of the chemical composition of the nerve fibre's different constituents have been filled, can the significance of these submicroscopic structures be properly evaluated. The great difficulties which beset a direct chemical analysis of the lipid and protein constituents of the axon will make it necessary to apply a combination of different cytochemical procedures. Among these, x-ray absorption microradiography as already applied by ENGSTRÖM and LÜTHY[35] on single fibres appears to be particularly promising, in combination with selective extraction and enzyme digestion.[59] Localization of important inorganic constituents in different regions of the nerve fibre may be possible by micro-incineration, combined with parallel micro-chemical analysis and electron diffraction investigations. Fresh nerve sections are especially suitable for these studies since a correlation can be obtained with the submicroscopic morphology of the object.

It would obviously be premature to discuss the bearing of this general picture of nerve fibre structure on the problem of impulse

propagation in nerve. However, in view of the coaxial arrangement of multiple membranes which these studies primarily suggest, it may be of interest to call attention to another aspect of the highly ordered, fluid-crystalline structure of the myelin sheath. Thus, if we look upon myelin mainly as a fluid crystal[69] it may not be too speculative to assume that it could also exhibit certain specific properties of crystals such as piezoelectric effects, semiconductor properties, etc. or equivalent phenomena.[43] In particular, the concepts of electron mobility and magnetic concentration of holes and electrons which are evolving from the field of semiconductor research[123] may eventually prove to be of operational value for an understanding of the electrical properties of the myelin sheath.[54] While these concepts are of course not directly applicable to the smectic fluid-crystalline state of the myelin layers, they may nevertheless induce new experimental approaches to the study of membrane properties and their relation to bioelectric potentials.[82] Solely as a working hypothesis these speculations on crystal properties associated with the fluid-crystalline nature of myelin might therefore prove interesting.

Considering the unique properties of nerve, it is apparent that what ultra-structural analysis has revealed up till now is but a glimpse of the extraordinarily complex organization which these glistening threads still hold in store for us.

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THE NUCLEOPROTEIN COMPLEX OF THE CELL NUCLEUS, AND ITS REACTIONS

P. F. Davison, B. E. Conway and J. A. V. Butler

I. The Isolation of the Nucleoproteins of Cell Nuclei

The nucleoprotein complexes of the cell nuclei have been studied intensively in the seventy years since Miescher began his classical researches. The basic extraction procedures, however, have undergone little change. Miescher[1] in 1874, obtained a protamine from fish sperm by acid extraction, and Kossel, in 1884, in a similar manner obtained a histone from goose erythrocytes.[2] The extraction of intact nucleoprotein from calf thymus, by water, and its precipitation by salt, was effected by Bang,[3] in 1899, and Huiskamp slightly modified the procedure in 1901.[4] In essentials these techniques have scarcely been improved.

The usual methods for isolating nucleoproteins depend upon their solubility properties, which are distinct from those of most other complex constituents of the cell. Calf thymus deoxyribonucleoprotein, for example, is insoluble in solutions of ionic strength between 0·02 and 0·5 (Mirsky and Pollister[5] and Luck, Eldredge and Kupke[6]). At lower and higher ionic strengths, and about neutral *p*H, it will dissolve completely in many solutions. The soluble cell constituents, including the ribonucleoproteins, may therefore be washed away in 0·14 M sodium chloride solution, and by selecting a suitable solvent, the deoxyribonucleoprotein (DNP) may be extracted from the cell debris. Stern and Davis[7] dissolved the DNP from the insoluble residues with water. Electrophoretically this material was inhomogeneous, but by adding salt to bring the concentration in the solution to 0·14 M the DNP was precipitated. When this precipitate was redissolved in water a more homogeneous product was obtained. Although it has usually been assumed that DNP is physically homogeneous, there is no great justification for this assumption, and the "purification" may involve selection of one fraction of the complex. Bernstein and Mazia[7a] found that such a procedure was accompanied by degradation with sea urchin sperm nucleoprotein. Mirsky and Pollister,[8] and many workers after them, have extracted the DNP from the cell debris by molar saline solution. As is discussed below, in a solution of high ionic strength most nucleoprotein complexes are largely dissociated. By

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diluting the solution with a large volume of water MIRSKY and POLLISTER lowered the ionic strength, and the DNP complex precipitated in the form of long fibres. However, obtained in this way, the nucleoprotein is a reconstituted complex differing in physical properties from the DNP extracted by water. MIRSKY and POLLISTER,[5] using the Dische reaction,[9] estimated all the nucleic acid present in the material to be deoxyribonucleic acid (DNA), but MAURITZEN,[10] working on DNP prepared in a similar manner, analysed the nucleic acids by the method of ROY, STEDMAN and MAURITZEN[11] and demonstrated that the complex was appreciably contaminated by pentosenucleic acid (PNA). BUTLER, DAVISON and JAMES[12] have prepared calf thymus DNP by a method similar to that of STERN and DAVIS,[7] and, using the analytical procedure of SCHMIDT and THANNHAUSER,[13] have found a significant proportion of PNA to be present in the nucleoprotein.

To reduce the degradation of the DNP in the course of extraction, the whole procedure is carried out at temperatures below 5°C, and several workers have employed depolymerase inhibitors. For this purpose EULER *et al.*[14] used fluorides, STERN *et al.*[15] used arsenates and PETERMANN and LAMB[16] used citrates in the isolation media. WEBB[17] has pointed out, however, that these ions have only been shown to inhibit *pancreatic* deoxyribonuclease. This enzyme has an optimum activity at *p*H 7. WEBB,[17] and ALFREY and MIRSKY[18] have studied the intracellular deoxyribonucleases, and have shown that they are not inhibited by the ions mentioned above. These enzymes have a maximum activity about *p*H 5·3 and are almost inactive above *p*H 7. The inhibiting effect detected in the experiments of PETERMANN and LAMB, possibly resulted not from a specific ionic effect, but from the capacity of the salt they used to raise the *p*H of the tissue homogenate, which is normally about 6·5, to a value where the depolymerase activity was very low. However, SHACK and THOMPSETT[55] have reported serum deoxyribonuclease to behave similarly to the pancreatic enzyme, although they confirm the observations of MAVER and GRECO[56] and the authors mentioned above, on the intracellular nucleases. Thus citrate and similar inhibitors might be effective in homogenates of tissues with a high blood content.

FELIX and HARTENECH,[19] MAVER and GRECO,[20] and FEINSTEIN,[21] among others, have prepared deoxyribonucleoproteins by different techniques, but none of their methods appears to offer any marked advantage over the more usual procedures. ORGEL and STERN[22] have briefly reported a very considerable elaboration of the water extraction technique employing buffered reagents.

In an attempt to obviate the danger of cytoplasmic contamination, many workers, including LUCK[23] ZBARSKII and DEBOV,[24] and EULER and HAHN[14] have isolated the nuclei from the cells, prior to the

extraction of the DNP. The material obtained in this way may still be contaminated by other nuclear contents, including PNA, however, and by components of the cytoplasm which may be adsorbed on the nuclei in the process of isolation (NEFF,[25] WEBB[17]). Also, as EULER and HAHN[26] have reported, the DNP from such isolated nuclei may be more degraded than that extracted directly from the whole tissue homogenate. It is probable that cathepsins, which MAVER, GRECO, LØVTRUP and DALTON[27] have shown to be present in cell nuclei, left active by the relatively mild procedure used in the isolation of the nuclei, may partially digest the protein moiety of the DNP complex. Despite earlier reports to the contrary, MAVER and GRECO[20] have shown that cathepsins can degrade protein even when it is in combination with DNA. FRUTON, IRVING and BERGMANN[28] isolated some intracellular cathepsins and showed that many of them are typical thiol-reactive enzymes. The activity of these proteases might be reduced by including mercuribenzoate or ethyl iodoacetate in the isolation media. In related experiments BUTLER, DAVISON and JAMES[12] found such inhibitors to exert a considerable effect on calf thymus cathepsins. BROWN, JACOBS and LASKOWSKI[29] have also reported that there are deoxyribonucleases in the nucleus, and these might depolymerize the DNA in the slightly acid media commonly employed for the isolation of nuclei. However SCHNEIDER and HOGEBOOM[29a] decided that the intracellular deoxyribonuclease activity was characteristic of the mitochondria, and WEBB[17] considered that these depolymerases were only adsorbed on to the nuclei in the course of their isolation in ionic solutions. Even if this is the case, it is well known that the nuclear membrane, in isolated nuclei at least, is readily permeable to many proteins, and so these enzymes could still attack their substrate.

BEHRENS[30] devised a method of fractionating powdered, freeze-dried tissue in organic solvents and so isolating the nuclei. DOUNCE, TISHKOFF, BARNETT and FREER,[31] and ALFREY, STERN and MIRSKY,[32] have developed the technique. By this means an ungraded DNP complex might be isolated from nuclei, free of cytoplasmic contamination. However, WHITE and ELMES,[33] and LUCK *et al.*,[34] have reported changes in the physical properties of DNP after freeze-drying. BEHRENS and TAUBERT[57] have also dehydrated the tissue by acetone but changes in structure would still be expected in such a highly hydrogen-bonded structure as the DNP complex is thought to be.

Thus in spite of intensive investigations there are no experimental procedures by which an undamaged deoxyribonucleoprotein free of adsorbed impurities may be extracted from cell nuclei. The use of specific enzyme inhibitors might improve the preparations, and the use of glycine as a nucleoprotein solvent, as suggested by STERN and DAVIS,[7] might also facilitate the extraction of a purer nucleoprotein.

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Deoxyribonucleoproteins are ill-defined complexes, and it is very difficult in a critical analysis to differentiate between the bound components and adsorbed contaminants. The gross chemical analyses reported in the literature show a wide variation. The nitrogen to phosphorus (N/P) ratio of the material roughly indicates the ratio of nucleic acid to protein, for whereas the nitrogen content of both proteins and DNA are similar, the DNA contains about 9% phosphorus while the proteins contain very little or none. The N/P ratios of the soluble deoxyribonucleoproteins are usually reported to lie between 3·4 and 4·0, but variations are found not only when different preparative techniques are compared, but also between individual samples from the same tissue prepared by identical methods. The DNA usually comprises 40 to 50% of the nucleoprotein, but ORGEL and STERN[22] have reported the low figure of 20%. GREENSTEIN and JENRETTE[35] reported the isolation of certain nucleoproteins with an N/P ratio of 15, but MIRSKY and POLLISTER[5] have shown that 75% of the nucleic acids of these preparations was PNA.

MIRSKY and RIS[36] have pointed out that some of the protein more loosely bound to the DNA may be lost in the process of isolating the DNP. It would appear that it is not possible at present to determine the original structure and composition of these labile complexes.

MIRSKY[37] has applied anaphylactic and immunological tests to prove the absence of cytoplasmic components in some nuclear preparations. However, such tests are limited in interpretation.

Solubility of Deoxyribonucleoproteins—The characteristic solubility properties of calf thymus nucleoprotein are similar to those of nucleoproteins prepared from many animal somatic tissues. These solubility properties slowly change when the complex is kept in solution (BERNSTEIN and MAZIA,[7a] GAJDUSEK[38]), and the nucleoprotein cannot then be precipitated by 0·14 M salt solution. These changes probably result from alterations in structure brought about by enzyme action. However, the deoxyribonucleoproteins from the sperm heads of many animal species show anomalous solubility properties.

POLLISTER and MIRSKY[39] reported that whereas trout, shad, mouse and frog sperm nuclei would dissolve in molar saline, and sea urchin, key-hole limpet and fresh-water clam sperm would dissolve in 2M saline, bull sperm heads were insoluble in salt solution of all concentrations. DALLAM and THOMAS[40] studied bull and boar sperm proteins and showed that a lipoprotein could be extracted by 0·14 M salt at *p*H 9. By extraction with sodium hydroxide and isoelectric precipitation, at *p*H 11 a basic protein was obtained, and at *p*H 2 a nucleic acid fraction. Probably at the high *p*H of the alkali used, the charge on the basic protein was reversed, dissociating the insoluble DNP complex and allowing the components to dissolve separately. MIRSKY and POLLISTER

advanced the idea of this mechanism to explain the dissolving of a nucleoprotein complex in 0·14 M salt, when the *p*H was raised above the iso-electric point of the protein.[5] Intact nucleoprotein has not been obtained from bull or boar sperm heads.[40a] Many sperm nucleoproteins are insoluble in water.

II. Physico-chemical Properties of Nucleoproteins

The work so far carried out on the intact nucleoproteins of the nucleus can only be regarded as of a preliminary nature. It is much complicated by the peculiar solubility behaviour of these substances, owing to which they can only be studied in solution either at very low ionic strengths or at high ones.[101] Under the latter circumstances, there is much evidence that the nucleoprotein is dissociated largely or completely into nucleic acid and protein. The fact that such dissociation occurs was indicated by the marked increase of viscosity in strong salt solutions as compared with water which often occurs. Mirsky and Pollister[41] who were originally under the impression that molar sodium chloride solution extracted a native nucleoprotein, later realized that in such solutions dissociation occurs. They showed that after sedimentation of the nucleoprotein solutions at 35,000 r.p.m. for 5 hours in 1 M sodium chloride, marked changes of the protein/nucleic acid ratio occurred at different levels of the cell, the upper layers containing a large excess of protein. Stern[42] also showed that sedimentation of such solutions alters the nucleic acid/protein ratio at different levels and concluded that the nucleic acid present is largely if not entirely free. Similar experiments were made by Petermann and Lamb[16] on a beef spleen nucleoprotein, with similar results.

The dissociation of calf thymus nucleoprotein into two components has also been observed in the analytical ultracentrifuge by Butler and Gilbert.[43] Their sedimentation diagrams in N sodium chloride show a very sharp rapidly moving boundary, of the same character as that of nucleic acid and a slow and rather diffuse boundary, due to substances of comparatively low molecular weight, presumably the dissociated proteins (see Fig. 1*A*). It is rather difficult on a preparative scale to obtain the supernatent solution from nucleic acid, but the proportion of protein is greatly increased (Fig. 1*B*).

Such experiments do not indicate clearly whether the dissociation of the nucleoprotein complex is complete. The occurrence of a single boundary is not evidence of the homogeneity of either nucleic acid or of nucleoprotein, as even artificial mixtures of nucleic acid and nucleoproteins give a single sharp boundary, owing to the fact that elongated fibres felt together and carry each other down. For this reason electrophoretic evidence of homogeneity of such preparations must be regarded as very doubtful, although no doubt a complex picture must

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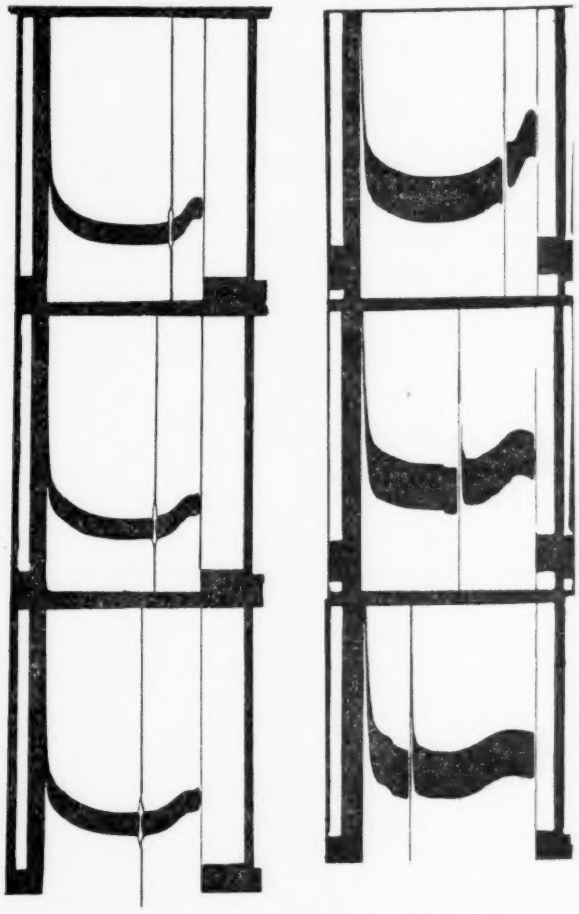
indicate heterogeneity. WINKLE and FRANCE,[44] for example, studied the rabbit liver nucleoprotein, made by the method of MIRSKY and POLLISTER both at low ionic strengths and in 1 M sodium chloride. In



A *B*

Fig. 1. Sedimentation pictures showing dissociation of thymus nucleoprotein in 10% sodium chloride solutions (successive pictures are at intervals of 15 mins. Speed in Spinco ultra-centrifuge, 60,000 r.p.m. (BUTLER and GILBERT[43]). (*A*) Original thymus nucleoprotein. (*B*) Supernatent fraction from a partial separation of protein and nucleic acid effected by centrifuging.

both cases they obtained complex electrophoretic patterns, which they regarded as evidence of heterogeneous character of the material. FRICK[45] has also studied thymus nucleoprotein in 1 M sodium chloride and has obtained complex patterns indicative of at least three

components. A recent study of this type has been made by FLEMING and JORDAN[46] who found two main boundaries, of which the slower was certainly complex, in solutions of high ionic strength and also two boundaries of which the faster was complex at low ionic strengths. They considered that the dissociation of nucleoprotein was usually incomplete and gave rise to a fast component with a relatively high nucleic acid content and a slow component which was either a mixture of nucleoprotein and protein, or purely protein according to the *p*H. This was supported by the analysis of fractions taken from the various boundaries. A similar report has been made by COOK.[83]

FRICK studied the precipitation of thymus nucleoprotein on dilution of a solution in molar sodium chloride. The precipitation curve is discontinuous and indicates that the material is heterogeneous. The intrinsic viscosity of the nucleoprotein remaining in the solution at various salt concentrations changes little above 0·6 M sodium chloride; below that there was a marked drop, but the concentration also diminishes rapidly in this region. This might indicate that the particles with the highest intrinsic viscosity in salt solutions (i.e. largest size) are precipitated first. The effects could, of course, also be due to a higher intrinsic viscosity of the dissociated nucleic acid than the reformed nucleoprotein.

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Another method of studying the dissociation of the nucleoprotein was used by BUTLER, DAVISON and JAMES[12] who found that on adding alcohol or acetone to the strong saline solution of nucleoprotein, most of the protein remained in solution, while the nucleic acid precipitated is comparatively free from protein. The amount of protein carried down by the nucleic acid depends on the salt concentration. From a 10% sodium chloride solution a fairly uncontaminated nucleic acid is obtained (N/P = 2·3). If this precipitate is redissolved in the salt solution and reprecipitated the N/P ratio is reduced to 1·9 (pure DNA, N/P 1·7). From this it appears that the amount of protein in thymus DNP which cannot be dissociated by 2 M sodium chloride is quite small, it is probably bound to the nucleic acid in a different way and may be residual pentose nucleoprotein. The dissociation is certainly incomplete in 1 M sodium chloride, and the observations of VAN WINKLE, mentioned by RABATIN *et al.*[58] confirm this.

LUCK and co-workers[59a] have reported a nucleoprotein complex obtained in good yield from rat liver nuclei which does not dissociate in strong salt. The physical examination of this material will be awaited with interest.

It is evident that physico-chemical studies of the intact nucleoprotein can only be made in the very limited range of low salt concentrations in which the material dissolves. Not much work has been done on preparations which have never been exposed to concentrated salts.

CARTER and HALL[47] examined water extracted protein from calf thymus and hog thyroid and found them to be electrophoretically homogeneous. Their sedimentation and diffusion constants were however determined in concentrated salt solutions and do not refer to the undissociated nucleoprotein. GAJDUSEK'S water extracted preparations[38] also gave only one peak in electrophoresis in buffers from *p*H 5–10, and after treatment with 1 M sodium chloride was multi-disperse.

There are conflicting reports about the viscosity of water-extracted nucleoproteins. Some workers have succeeded in obtaining highly viscous preparations e.g. PETERMANN and LAMB[16] reported that water extracts of washed thymus nuclei had viscosities as high as similar solutions of DNA. AHLSTROM, v. EULER and HAHN[16a] also obtained a similar preparation; but others have obtained nucleoprotein in a state having a much lower viscosity than that of nucleic acid at the same concentration. There are similar differences in the solubility in salt solutions, and as a rule the product becomes less easily precipitated by salts on keeping, probably owing to enzyme action.

The most thorough recent study of water extracted material is that of STEINER.[48] His material sedimented as a rather broad peak, in marked contrast to sodium thymonucleate, with sedimentation constants which were not greatly dependent on the concentration. The viscosity of 0·2% solutions in 0·015 M phosphate buffer was however low and shear independent, which indicates that a considerable amount of degradation or at least diminution of asymmetry had taken place during the preparation. From a study of the light scattering, STEINER arrived at the conclusion that the average molecular weight of his preparation was 5,800,000; with a particle length of about 3000 A and an axial ratio of only 24. It must be observed however that the solutions used by STEINER had been subjected to a very thorough clarification treatment, by taking up the precipitate formed with 0·14 M salt solution in a large volume of water and subjecting it to centrifugation at 15,000 r.p.m. in a Sorval centrifuge. By three repetitions of the process all the heavier particles of the nucleoprotein must have been removed and STEINER'S work therefore probably refers to the smaller particles present. SHOOTER, DAVISON and BUTLER[49] found that with a nucleoprotein solution which had not been subjected to this extensive clarification procedure, there was a considerable proportion of a very rapidly sedimenting gel-like component, which gave a boundary which gradually sharpened during its passage down the ultra-centrifuge cell. It was followed by a slower boundary, moving with approximately the same speed as that observed by STEINER. It would appear from this that the aqueous extracts contain "islands" of gel-like material, together with some more highly dispersed particles. It is not known whether the gel-like material can be dispersed without essential injury.

Some work of this kind has also been done on "reconstituted" nucleoproteins prepared by extraction with concentrated saline (MIRSKY and POLLISTER). WINKLE and FRANCE[44] estimated the molecular weight of a preparation from rabbit liver by sedimentation and diffusion in 0·02 M phosphate to be $1{\cdot}6–2{\cdot}5 \times 10^6$; with an axial ratio of 40–60.

The nature of such "reconstituted" materials is however very uncertain. From the fact that when deoxyribonucleic acid (thymus) and protamine are mixed an insoluble precipitate is formed as indeed also occurs with the nucleic acid and isolated histones, ALEXANDER[50] suggested that the insolubility is due to the ability of the basic proteins to combine with and so cross-link two or more nucleic acid particles. The same should be true of reconstituted nucleoprotein made by dilution or dialysis of a concentrated salt solution. It is however found that although precipitation occurs at first, on continued dialysis the nucleoprotein may go into solution, or at least into a gel-like state, again. An artificial "nucleoprotein" may also differ from a native material in that the molecular configuration of both the nucleic acid and the protein components may be greatly modified in the isolation procedures. For all these reasons it is doubtful if reconstituted and artificial nucleoproteins have more than a superficial resemblance to the native material. Both water extracted and "reconstituted" nucleoproteins undergo changes of solubility even on freeze drying. AMBROSE and BUTLER[51] found that solid films of thymus and herring sperm nucleoproteins, made by freeze-drying thin layers of their aqueous solutions, did not completely redisperse on being placed in water, but swelled considerably and, in many cases, the swollen gel remained in apparent equilibrium indefinitely. This is probably due to the fact that when all the water is removed from the nucleoprotein preparation, hydrogen bonding can occur between adjacent fibres; thus converting the material into a cross-linked gel, which imbibes water so far as the cross-links permit.

These fibres can be obtained in an orientated state by forming them on a thin rubber basis and then stretching the rubber in one direction while they are moist. After drying the rubber it is removed by a suitable solvent and the fibres remain in a partially orientated condition, as can be shown by the dichroism they exhibit. In water an overall swelling occurs, but it is remarkable that a tendency to return to the original shape persists, showing that the original stretching has been done against elastic forces which remain in the solid film.

The swelling of these films is reduced by quite low concentrations of salts, particularly by salts with multivalent cations. It was found that the concentration of salt which just inhibits swelling is of the same order as that which is just sufficient to precipitate the nucleoprotein

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from moderately concentrated solutions. Polyvalent cations may also cause a marked shrinkage of the films when present at somewhat greater than the inhibiting concentration. This behaviour is very similar to the shrinkage of nucleoprotein drops observed by JEENER,[52] when placed in solutions of various ions. The relative concentrations of different ions which inhibit swelling and cause precipitation are not unlike the lyophobic series of concentrations which precipitate a negatively charged colloid, and it may therefore be expected that these effects are due to the reduction of the net negative charge on the particles when positive ions are adsorbed. Heavy metal ions, e.g. Ag^+, have a greater effect than corresponds with the charge and it was suggested that these ions complex with the $—NH_2$ groups of the nucleoprotein.

AMBROSE[51] determined the infrared dichroism of orientated sheets of dried nucleoprotein prepared as described above. Absorption bands at 1695 cm^{-1} and 1655 cm^{-1}, which are assigned to stretching vibrations of the CO and CN bonds of the purine and pyrimidine rings show a maximum absorption with the E-vector of the plane polarized radiation perpendicular to the fibre axis. This is similar to the finding of FRASER and FRASER[53] in well-orientated nucleic acid fibres and is interpreted as meaning that the purine and pyrimidine rings lie at right angles to the direction of stretching i.e. at right angles to the fibre axis. In the case of nucleoprotein there are also absorption bands not present in the nucleic acid and at 1640 cm^{-1} and 1540 cm^{-1}, which suggest that the protein is present in the β-configuration and the dichroism in the stretched samples suggest that these chains are parallel with the nucleic acid fibre axis. It is also possible however that the protein is in the α-form and orientated at right angles to the fibre axis. FRASER[54] has pointed out that similar results are obtained with intact octopus sperm heads.

Nucleoprotein in the living cell

It is not certain that DNP occurs in the living cell as a preformed complex. According to CASPERSSON,[115] his microspectrophotometry of living cells has revealed a constant change of the proteins associated with the DNA throughout the mitotic cycle. DOUNCE[107] has suggested that if the histone is bound to DNA in the nucleus it does not block all the phosphate groups, for on lowering the *p*H of the nucleus below the iso-electric point of some of the other proteins of the cell they combine with the DNA. This combination results in a shrinkage of the nuclear contents from loss of the water molecules about the polar groups. DOUNCE also reported a difficulty in dissolving out DNP from freshly isolated nuclei by molar saline, although the solvent readily dissolves extracted nucleoprotein. He suggested that slight autolysis is required to render the DNP soluble. However, in contrast, POLLISTER

and MIRSKY[39] reported that strong saline immediately caused the nuclei of fresh sperm to swell, suggesting the DNP readily dissolved.

Several authors have reported the isolation of fibrous structures from cell nuclei. These structures contain DNP, and, if the fibres are not artefacts, it would seem probable that the complex is present preformed in the cell. CLAUDE and POTTER[116], by sand-grinding leukemic cells obtained "chromatid threads" containing 40% DNA. GOPAL-AYENGAR and COWDRY[117] obtained similar fibres which they identified with the interphase chromosomes. After dissolving away the DNP with water or molar saline they found refractile granules were left. MIRSKY and RIS[36] have claimed the isolation of intact interphase chromosomes, from which histones could be extracted by molar salt solution containing 1% citric acid. By displacement studies with dyes and protamines they showed that in these fibres histone is definitely combined with DNA. By dissolving out the DNP by salt they found a "residual chromosome" which they showed was the structural skeleton of the "chromosome." MAZIA and co-workers[118], [119] had reported similar findings after enzyme studies on drosophila chromosomes, although KAUFMANN, GAY and MCDONALD[120] more conservatively suggested the chromosome was an integrated structure. However the conclusions reached from the enzyme studies closely parallel those reported by MIRSKY and RIS. The latter authors also claimed that their "isolated chromosomes" were very similar to the state in which they occur in the nucleus, but they seemed to have no place for the nuclear sap. BROWN, CALLAN and LEAF[121] have confirmed the existence of nuclear sap. LAMB,[122] and CALVET, SIEGEL and STERN[123] have disputed the identity of these "isolated chromosomes" but recently DENUES[124] offered evidence to support the view that those fibres are identical to the chromosomes visible in the dividing cell.

Although these isolated nucleoprotein structures may be in a state closely similar to that in which they exist in the living cell, the possibility must always be borne in mind that much rearrangement may occur among the nuclear components on the death and disruption of the cell. Different workers have suggested various criteria for assessing the effect of the isolation procedure on the cell structures. SCHNEIDER and HOGEBOOM[125] considered an "undamaged" nucleus should appear homogeneous under the microscope. DOUNCE[31] has suggested that the chromatin network may be visible in undamaged nuclei, and that damaged nuclei, in which slight autolysis might have occurred, might be recognized by their failure to gel in dilute alkali or saline. CUNNINGHAM, BROWN, LASKOWSKI and IHRIG[126] have isolated thymus nuclei by an exceptionally mild technique, but recognized that some change probably occurred on the disruption of the cell. Visible changes take place in the nucleus of an amoeba on enucleation or on the death of the cell, and

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such changes probably occur to some degree in all cells. Thus only cautious and conservative conclusions should be drawn from the properties of any isolated cell component and the possibility that deoxyribonucleoproteins are only necrotic artefacts should not be forgotten.

III. The Components of the Nucleoprotein Complex

A. *The proteins of the nucleus*

As it is difficult, if not impossible, to be sure that some of the proteins, bound to the DNA originally, are not washed out by the isolation procedure, it is proposed briefly to discuss all the proteins that have been extracted from the nucleus. Since many more studies have been made of the nuclear proteins than of the components of previously extracted nucleoprotein, the nature of the proteins of this complex might be more completely understood by this wider approach. It is sometimes assumed that the basic proteins are the only proteins combined with DNA, but Mirsky and Pollister,[5] Hamer,[60, 60a] Khouvine and Gregoire[61] and Bernstein and Mazia[7a] have reported a protein fraction in DNP other than the basic protein. Of the nature of this second protein fraction nothing is certain.

The major protein combined to the DNA in the nucleoprotein complex is certainly a basic protein, either a histone or a protamine. These basic proteins, which appear to be confined to the nucleus of the cell, exhibit very distinctive physical properties, the most noteworthy of which being the very high iso-electric point. No metabolic function has yet been proved for these proteins, although Stedman and Stedman have suggested they might serve as mitotic regulators,[62] or to modify the gene function in the differentiated cells.[63]

The protamines, relatively simple proteins of low molecular weight, obtained from sperm cells of many species of fish, are strongly basic and form salts with many acids. They are usually extracted by sulphuric acid, or hydrochloric acid, although Kossell[64] has also employed copper chloride. From the acid solution, the protamines may be precipitated by ethanol, when they form an oil which solidifies when it is treated by more ethanol. The protamine sulphates are white powders which readily dissolve in water or dilute acid. Felix and Dirr[65] have isolated the protamines as picrates from the acid solution. By suspending the protamine picrates in methanol and passing in hydrogen chloride gas the methyl ester hydrochlorides were obtained. Some of the more intensively studied protamines are salmine from salmon sperm, clupein from herrings, sturin from sturgeon and scombrine from mackerel.

From analytical results the molecular weights of several different

protamines have been inferred to lie between 4000 and 10,000 (RASMUSSEN and LINDERSTRØM-LANG,[66] TRISTRAM[67]). Since protamines will dialyse through cellophane, FRAENKEL-CONRAT and OLCOTT[68] and POLLISTER and MIRSKY[39] have suggested similar figures. ANDO and HASHIMOTO[69] have also reported the dialysis of a protamine, but until proteolysis has been tested for and disproved, no conclusions should be drawn from this behaviour. Protamines are characterized by a very high proportion of arginine which sometimes accounts for more than 90% of the protein nitrogen. Their nitrogen content is high, as a consequence, the usual figure being well over 20% compared with about 16% nitrogen for most proteins. Relatively few amino-acids have been reported in protamines and, except for the reports of DUNNE,[70] and a few early workers whose analytical procedure was of limited accuracy, no aromatic amino-acids have been found. The ultraviolet spectra of protamines, therefore show no absorption about 275 mμ, and the proteins do not react with Millon's reagent.

Histones are also strongly basic proteins with an iso-electric point about *p*H 11.[5] They are distinguished from protamines by a lower nitrogen content, usually about 17%, and they are precipitated from solution by ammonium hydroxide. They are distinguished from other proteins by their solubility in a hot or cold solution of sulphuric acid containing mercuric ions (DALY, MIRSKY and RIS[71]). Histones have a high lysine and arginine content, but they contain almost all the usual amino-acids. They will not dialyse through cellophane, but KHOUVINE[72] has reported that they will dialyse through soft collodion. From analytical figures the molecular weights of histones have been estimated to lie between 14,000 and 20,000 (BRUNISH, FAIRLEY and LUCK[73]; FELIX and RAUSCH[74]).

Histones have been shown to occur in the somatic tissues of most animals, and in many sperm cells. They are extracted from the nuclei or nucleoprotein by various acids. The histones are precipitated from acid solution by ethanol, by acetone, by the addition of alkali to *p*H 11, or by salting-out with sodium chloride. HAMER has isolated the histones in the form of complexes with detergents[75].

Protamines were thought to occur only in the ripe sperm cells of fishes, and, since histones were isolated from the unripe testes it was suggested that histones were the precursors of protamines in the cell. However STEDMAN and STEDMAN[76] have shown that the nitrogen contents of the basic proteins of salmon sperm, liver and erythrocyte nuclei are all different, the sperm protein being a protamine, the liver and erythrocyte nuclear proteins being histones. Moreover the STEDMANS have reported a differentiation between the *histones* isolated from different tissues of the same animal. They have pointed out that since the basic proteins are specific to the tissues of the animals they cannot

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be identified with the genes. No cell nucleus has been shown to contain both a histone and a protamine, and the STEDMANS[63] suggest that the two classes of protein serve the same function in the cell nucleus. Thus a sharp distinction between the protamines and the histones would not appear to be justified, and it is not unexpected that the isolation of proteins with properties intermediate between the two classes have been reported. DUNNE, and a few early workers have reported protamine-like material containing aromatic amino-acids. HULTIN and HERNE[77] have analysed basic proteins extracted from the sperm of sea-urchins and limpets. For these proteins the authors estimated molecular weights between 14,000 and 20,000 but the amino-acid proportions were typical of neither the histones nor protamines. DALY, MIRSKY and RIS[71] have described a protein isolated from rooster sperm which they called Gallin. This protein, with a high arginine content, and containing no lysine, they considered to be a protamine. However it resembled a histone in containing most of the common amino-acids, including tyrosine.

A large number of analyses of protamines and histones have been reported. (Clupein, see refs. (14), (78), (79), (80), (81) and (82); salmine, see refs. (64), (67), (78), (82), (84), (85); histones, see refs. (60), (71), (73), (86), (87), (88).) However, the analyses show variations between one report and another, larger than the estimated experimental errors, and DALY, MIRSKY and RIS[71] and HARPER and MORRIS[88] have remarked on the variations in the analyses of different samples of their own preparations. To explain these differences it has been suggested that in any one tissue the basic proteins are not identical, but that they are a mixture of proteins with very similar properties, from which even minor differences in experimental technique will cause different fractions to be extracted. KHOUVINE and BARON[89] have reported a greater difference between the histones from rat epithelioma nucleoprotein extracted by sodium chloride on one hand, and by calcium chloride on the other, then between the histones obtained from rat epithelioma and calf thymus by a common technique. CARDOSO and PIRRO[90] have also pointed out the dependence of the analytical data on the method employed in the preparation of the protein material.

The evidence on the occurrence of tryptophane and the sulphur amino-acid in histones is at present conflicting. STEDMAN and STEDMAN[76] have used the absence of tryptophane in a histone preparation as a criterion of the purity of the extract. KHOUVINE and BARON,[89] ZBARSKII and PEREVOSHCHIKOVA,[91] HAMER,[60] and, in some early papers, MIRSKY and POLLISTER[5] have all found histones to be free of tryptophane. In later papers MIRSKY and RIS[92] confirmed the presence of tryptophane in small amounts, in histones, and DAVIDSON and LAURIE,[86] CARDOSO and PIRRO,[90] EULER and HOGBERG[93] and

ENGBRING and LASKOWSKI[94] have reported similar findings. HARPER and MORRIS[88] found tryptophane to be absent from chicken erythrocyte histone but present in liver histone. Of the sulphur amino-acids FELIX and RAUSCH[74] and the STEDMANS[63] have reported histone preparations giving the nitroprusside reaction for thiol groups, but the latter authors could detect no methionine. KHOUVINE and BARON[89] also found methionine was absent from their histone preparations. BRUNISH, FAIRLEY and LUCK[73] and HARPER and MORRIS[88] have detected methionine. HAMER[60] did not mention methionine but reported the absence of cysteine and cystine. EADIE and LEAF[87] decided that the sulphur amino-acids, if present, must be in very small proportions.

The only conclusion to be drawn from this conflicting evidence is that histones, as a class, are not characterized by the absence of any amino-acid except, possibly, cystine. It would appear, however, that certain histone fractions may be obtained which contain no tryptophane, or no methionine.

Although it has been suggested that the basic protein from any tissue are not homogeneous, few attempts at the fractionation of the histones or protamines have been reported. STEDMAN and STEDMAN[63] claimed the separation of certain histones and protamines into two distinct fractions by precipitation with ethanol. RASMUSSEN,[95] FELIX and DIRR,[65] and RAUEN, STAMM and FELIX[96] have fractionated clupein or clupein methyl ester, and shown the protamine to be complex. FELIX and co-workers[97] have reported salmine also to be complex.

From these reports it would appear that the heterogeneity of the basic proteins should be easily confirmed by physical investigations. RAUEN, STAMM and FELIX[96b] have shown clupein to be complex by electrophoresis, but the first physical studies of the histones that were made, usually suggested that they were homogeneous. HALL[98] found that calf thymus histone moved as a single component under electrophoresis. HAMER[60] reported a single experiment on thymus histone in the ultracentrifuge which revealed two components, but he suggested the heavier of these was denatured protein. BRUNISH, FAIRLEY and LUCK[73] found rat liver histone to be homogeneous in the ultracentrifuge.

However, AHLSTROM[99] reported a histone preparation, much of which dialysed through cellophane, and the non-dialysable fraction of which was complex under electrophoresis and ultracentrifugation. Recently LUCK, COOK and ELDREDGE[99a] reported the extraction of a histone preparation from calf thymus nuclei, isolated in glycol, of which up to 40% dialysed through cellophane. Enzyme inhibitors did not appear to prevent the appearance of this dialysable fraction. The

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non-dialysable fraction was separated into a light and heavy histone with sedimentation constants about 1·7 and 30 Svedberg units respectively. BUTLER, DAVISON, JAMES and SHOOTER[100] like HAMER[60] as mentioned above, found traces of a heavy histone present in this preparation. LUCK and co-workers ran the light histone for 200 minutes at 60,000 r.p.m. in a synthetic boundary cell, and still found the material homogeneous, but BUTLER and co-workers found that the light component split into two fractions of approximately equal proportions in prolonged runs under ultracentrifugation and electrophoresis. KHOUVINE, GREGOIRE and ZALTA[99b] have also split a rat epithelioma histone into two components by prolonged electrophoresis.

GREGOIRE, GREGOIRE and REYNAUD[99c] extracted histones by neutral procedures, avoiding the use of acid. The nucleoprotein was dissolved in strong salt and the DNA was precipitated by ethanol. From the supernate, which contained the protein in solution, the ethanol was removed by distillation *in vacuo* and the salt was removed by dialysis. A precipitate formed during the distillation which could be redissolved on prolonged dialysis against water. This fraction was homogeneous electrophoretically, and, like the heavy histone of LUCK and co-workers, was soluble with difficulty in phosphate buffers. The soluble protein fraction showed three components under electrophoresis. BUTLER, DAVISON and JAMES[12] in a similar series of experiments also obtained in insoluble fraction, but which could not be redissolved by any mild procedure. They found that the complexity of the soluble protein fraction varies considerably, and they concluded that proteolysis was occurring. By performing the whole preparation more rapidly and by including ethyl iodoacetate or an enzyme inhibitor in the media, they obtained histones resembling the acid extracted proteins more closely in physical behaviour. It is obvious that proteolytic degradation of the histone easily occurs and may be responsible for some of the contradictory findings. Extraction by acids clearly reduces the possibility of enzymic degradation but it must be remembered that the initial isolation of the nuclear material is usually made in neutral or only slightly acid media.

Although the degree of complexity of the basic proteins is still uncertain, it would appear that they do not form a wide range of closely similar proteins, but more probably a few different, and possibly labile, species. However, until a basic protein that has been conclusively shown to be homogeneous has been isolated, quantitative, and end-group amino-acid analysis[127][128] would appear to be premature.

Non-basic proteins of the nucleus

In 1942 MAYER and GULICK[102] isolated a previously unknown protein fraction from thymus nuclei obtained by the Behrens technique. This

fraction had an iso-electric point about *p*H 6. STEDMAN and STEDMAN,[103] in 1943 reported the detection of "chromosomin," an alkali soluble protein containing tryptophane and a large proportion of glutamic acid, in cell nuclei. ZBARSKII and DEBOV[24] reported a similar protein, along with another fraction which might have been denatured material. MIRSKY and POLLISTER[5] detected a tryptophane-containing protein in a nucleoprotein preparation, which they later thought was some of the "residual protein" from their "isolated chromosome" which had been taken up with the "nucleohistone" in strong salt solution. DALLAM and THOMAS[40] investigated a similar "acid" protein in sperm nuclei. WANG, MAYER and THOMAS[104] isolated a protein from liver cell nuclei and showed it to be a lipoprotein, while CARVER and THOMAS[105] studied it under electrophoresis and found it homogeneous. The identity of these proteins reported by the different authors mentioned is by no means certain. HAMER[60] separated the protein from DNP by the SEVAG[106] method, and reported that the non-histone fraction was distinct from "chromosomin" because it had a low glutamic acid content. It is also difficult to relate the properties of WANG's lipoprotein to those of "chromosomin." Whether or not these proteins are bound to DNA in the cell or in nucleoprotein is similarly doubtful, although the STEDMANS, and MIRSKY and POLLISTER have suggested that their respective proteins are bound.

ENGBRING and LASKOWSKI[94] digested chicken erythrocyte nuclei with deoxyribonuclease and from the digest extracted both a histone and a protein soluble in dilute sodium hydroxide. This latter protein showed an anomalous U.V. spectrum, and on hydrolysis, reducing sugars were detected in the hydrolysate. This protein fraction was shown to be heterogeneous. The fact that ENGBRING and LASKOWSKI had to degrade the DNA with a depolymerase before the protein could be extracted suggests that the protein is linked to nucleic acid in the cell, but whether the protein would appear in isolated nucleoprotein is uncertain.

ALFREY, STERN and MIRSKY,[32] and DOUNCE,[107] among others, have shown that the nucleus possesses considerable enzymic activity, as indeed its important role in the cell life shows it must have. DOUNCE[108] pointed out some years ago that some enzymes, for example arginase, appear to be bound in the nucleus, while others, for example aldolase, are easily washed out by aqueous reagents. Some of these more firmly bound enzymes are probably combined with nucleic acid and their enzymic activities might be detectable in the DNP, although if the binding is a non-specific attachment occurring on the death of the cell, no activity might be detectable, as OHLMEYER[109] has shown. Little work except that of STERN, GOLDSTEIN and ALBAUM[110] has been done on the enzymic activities of DNP. The correlation of enzyme activity with

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bound protein would have to be cautious, for BINKLEY[111] has demonstrated that some PNA has dipeptidase activity and it is not inconceivable that DNA itself may have enzyme properties. Some of the enzymes of the nucleus might also be present in the "acid protein" for MIRSKY and RIS[92] have found that all the alkaline phosphatase activity of the nucleus resides in the "residual chromosome," and cells with an active metabolism have a proportionality large "residual chromosome."[37]

A large fraction of the nuclear proteins are soluble in dilute saline, and some are extracted by water. BROWN, JACOBS and LASKOWSKI,[29] and DOUNCE[112] showed that protein was progressively lost as isolated nuclei were washed. POLLISTER and LEUCHTENBERGER[113] confirmed these observations by microspectrophotometry, although STEDMAN and STEDMAN[63] disputed this evidence. Recently KIRKHAM and THOMAS[114] have isolated the soluble protein from nuclei obtained by the Behrens technique, and shown it to be a globulin, homogeneous under electrophoresis. However, the ease with which this protein fraction is washed from the nucleus suggests that it cannot be bound to DNA in the cell and hence is unlikely to be detected in nucleoprotein. Probably many of the water soluble enzymes belong to this globulin fraction.

B. *Deoxyribonucleic acids*

1. *Composition of deoxyribonucleic acids*

It is appropriate to discuss here some recent work on the composition of DNA from different sources as this is of importance in considering the possibilities of specificity which they may possess.

A considerable amount of work on the relative proportions of the bases has been done since 1948, when three groups of workers viz., GULLAND and JORDAN[151]; CHARGAFF, VISCHER and ZAMENHOF[152] and HOTCHKISS[153] challenged the tetra-nucleotide hypothesis and questioned the occurrence of the bases in equimolecular proportions. This work was greatly helped by the development of paper chromatographic methods for determining micro-amounts of the bases by the CHARGAFF group. The results were definitely contrary to the equimolecular hypothesis, but CHARGAFF *et al.* found in all the cases examined and approximate equality of adenine and thymine and of guanine and cytosine, but in material from different sources very different proportions of the two pairs were observed. CHARGAFF's observations were defective in some cases in that up to 20% of the total phosphorus was unaccounted for in some cases. Part of this may be due to the presence of a fifth base, 5-methyl cytosine; isolated from deoxyribonucleic acid by WYATT[154] and COHEN,[155] but this is present in insufficient

amounts to account for the losses. WYATT's analyses[156] substantially confirmed CHARGAFF's[157] in most cases. The following table gives some of the results. It is to be noted that the wheat germ material contains, much more 5-methyl cytosine than that from any other source, and as it is associated with a low value for cytosine, it is possible that one may be substituted by the other.* Small quantities of uracil have also been reported. MAURITZEN[159] has pointed out that the determinations of DNA's from mammalian tissues are close to the proportions adenine 4, guanine 3, cytosine 3, thymine 4: but such proportions would only be significant if the DNA obtained from any tissue is itself homogeneous and not a mixture.

TABLE 1

Composition of desoxyribonucleic acid from various sources*

Source	A	T	G	C	MC	*Author*
	Mols of base/4·00 mols of P					
Human thymus	1·12	1·12	0·76	0·64	—	C
Calf thymus	1·13	1·11	0·85	0·85	·052	W
Locusta migratoria	1·17	1·17	0·82	0·83	·008	W
Bull sperm	1·15	1·09	0·89	0·83	0·052	W
Herring sperm	1·11	1·10	0·89	0·83	0·075	W
Wheat germ	1·05	1·08	0·94	0·69	0·23	W
Avian tubercle bacilli	0·48	0·44	1·12	1·04	—	C
Pig liver	1·04	1·04	0·76	0·76	0·20	M
Bacillus Schatz	0·72	0·64	1·04	1·16	—	C
Yeast	0·96	1·00	0·56	0·42	—	C
Salmon sperm	1·12	1·08	0·84	0·80	0·02	M
Salmon sperm	1·12	1·10	0·78	0·77	—	C. L. G. H.
Gypsy moth virus	0·845	0·80	1·225	1·13	—	W
Pine sawfly virus	0·29	1·21	0·785	0·715	—	W
Tent catapillar virus	1·18	1·11	0·91	0·80	—	W
M tuberculosis	0·72	0·78	1·16	1·35	—	W
E. coli	0·92	1·10	0·96	1·03	—	W

References: W[154] [156], C[155], M[158], C.L.G.H.[158]

* A = adenine; T = thymine; G = guanine; C = cytosine; MC = 5-methyl cytosine.

Evidence as to the order of the nucleotides in DNA has been obtained by the studies of the products of enzyme degradation by SMITH and MARKHAM.[160] The deoxyribonuclease digest of DNA contains di- and tri-nucleotides which can be separated by fractionation on ion-exchange resin columns. SMITH and MARKHAM treated the digest with a phosphomonoesterase, which removed the terminal phosphate groups from the polynucleotides and the resulting products could be separated by

* Yet another base, 5-hydroxy-methyl cytosine has been isolated by WYATT and COHEN from DNA of bacteriophage.[219]

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paper chromatography and by paper electrophoresis. In this way they obtained the following nucleoside esters from various DNA's A.C., A.T., G.T., C.T., C.C., T.T.; from wheat germ DNA, A.A., A.G., G.C., and G.M.C. (together) and from herring sperm DNA, a dephosphorylated tri-nucleotide containing A, C and T. The occurrence of these provides evidence of the irregular distribution of the bases in DNA.

ZAMENHOF and CHARGAFF[161] have also inferred a dissymmetery of the nucleotide sequence from the nature of the products of enzyme digestion. The distribution of purines and pyrimidines in both the dialyzable fragments and in the undialyzable core varies during the progress of the digestion.

They found that the purines can easily be removed from DNA by mild acid treatment, yielding apurinic acid. Degradation of this substance by alkali gives rise to a variety of dialysable and non-dialysable fragments, from the composition of which it is inferred that DNA consists of tracts consisting principally of pyrimidine nucleotides, as well as stretches in which purine nucleotides predominate.[165a–d]

CHARGAFF, CRAMPTON and LIPSHITZ[162] have obtained DNA fractions of different composition by a process of fractional dissociation of the nucleoprotein with varying concentrations of sodium chloride. Marked variations of the ratio of adenine and thymine to guanine and cytosine occurred in the different fractions. The fractionation appears to depend on differences in the strength of the bond between the nucleic acid and the protein. It appears from this that a number of distinct varieties of DNA, having different compositions exist, but A + T is always equal to G + C. The minor constituent 5-methyl cytosine was also present in different amounts in different fractions. BENDICH, RUSSELL and BROWN[163] have also separated the DNA of various organs of the rat into two fractions of differing solubility in 0·87% sodium chloride. In biosynthetic experiments with C^{14}-formate, it was found that C^{14} was incorporated to different extents in the two fractions, suggesting a different metabolic origin. BROWN and WATSON[165] have reported a fractionation of DNA in its passage down columns of cellulose covered with histone.

2. *Structure of the DNA particle*

The physico-chemical properties of these substances have been reviewed at length by JORDAN in Volume II of this series. (See also JORDAN[131] and SADRON[132]). Except in connection with the structures based mainly on information derived from x-ray diffraction pictures, there is not much new work to report.

RILEY and OSTER[133] have studied the x-ray diffraction of concentrated aqueous solutions (or gels) of DNA (2% to 100% by volume). In a narrow concentration region in the vicinity of 0·8 g/ml the material

appears to be fully crystalline. From 0·73 to 0·04 g/ml it possesses a partly-ordered structure of the "liquid-crystalline" type and in more dilute solutions there is no evidence of marked molecular ordering. In the micellar (partly crystalline) region two long spacings are observed, both of which increase with increasing water content. Two alternative explanations of this phenomenon are advanced. In the first, the spacings are ascribed (*a*) to an intramicellar parameter related to the distance separating neighbouring cylindrical molecules (*b*) the intermicelle separation. By extrapolation the diameter of the unhydrated single fibre is obtained as 16 A, and a micellar structure is proposed with 7 of these fibres in an approximately hexagonal array. The proposed micellar structure for the gel resembles that of a two-dimensional honey-comb and is not a simple space-filling model; as in tobacco mosaic virus. The second hypothesis is that the molecule is coiled into a helix, the shorter spacing corresponding to the pitch and the longer to the separation of neighbouring helices. On dilution, both spacings increase by virtue of the helix uncoiling.

New attempts have been made to propose a detailed structure of DNA in accordance with the facts. It will be remembered that Astbury[134] on the basis of a repeat distance of 3·4 A in the x-ray diffraction pattern of sodium deoxyribonucleate and its density suggested that the bases were piled on each other like a pile of pennies, with this separation. It was difficult to reconcile this with the spatial requirements of the sugar and phosphate groups, and alternatives have been suggested e.g. Furberg's zig-zag arrangements in which the bases stick out of the main chain on either side alternately with a separation on each side of about 7·0 A, and a spiral arrangement, in which six nucleotide units make a complete turn.[135] There was also a longer repeat spacing along the axis of about 30 A. Fig. 2 shows x-ray fibre pictures obtained by Franklin and Gosling.

A much more elaborate arrangement has been proposed by Pauling and Cory.[136] It consists of a triple helix, with the phosphate groups in the core and the bases disposed centrally around them. The axial distance between successive phosphate groups of one chain is 3·4 A. The phosphate groups of the three chains were arranged in groups of three, with six of the oxygen atoms (two from each phosphate) packed together to form a central octahedron. With three chains the repeat distance of the whole spiral is 27·2 A.

Alternative helical arrangements have been proposed by Watson and Crick;[137] Franklin and Gosling[138]; Wilkins, Stokes and Wilson.[137] Watson and Crick, on the basis of chemical evidence and models suggest a double helix (Fig. 3) in which the axes are composed of pairs of bases, adenine and thymine or guanine and cytosine, hydrogen bonded with each other, with the phosphate groups arranged radially.

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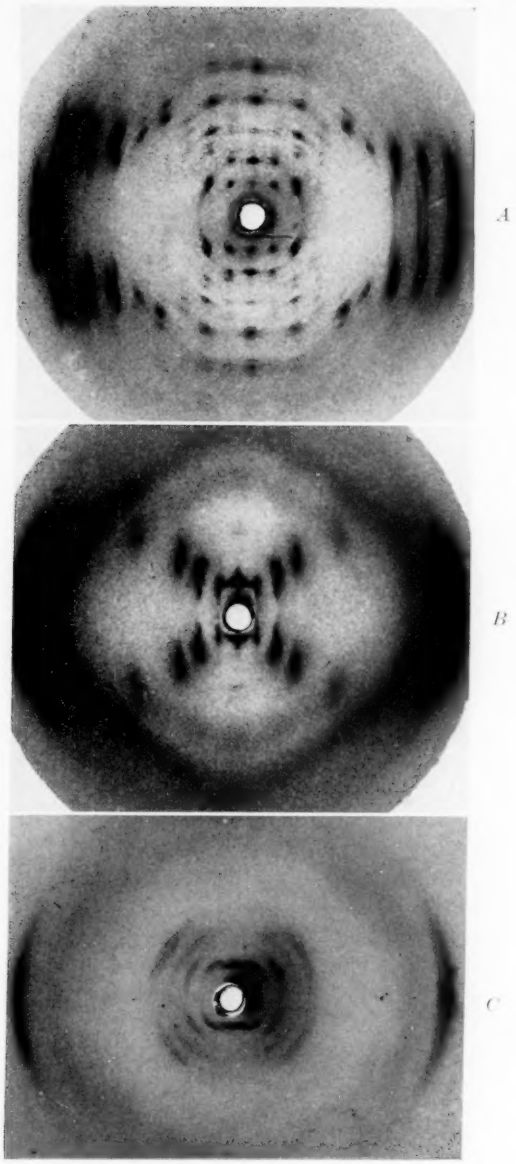

Fig. 2. X-ray fibre photographs of deoxyribonucleic acids. (*A*) Sodium thymonucleate (Signer preparation) giving "crystalline" fibre diagram. (FRANKLIN and GOSLING[138]). (*B*) Sodium thymonucleate fibre of high water content showing less ordered structure (FRANKLIN and GOSLING[138]). (*C*) *Sepia* sperm heads at 98% humidity (WILKINS and RANDALL[140])

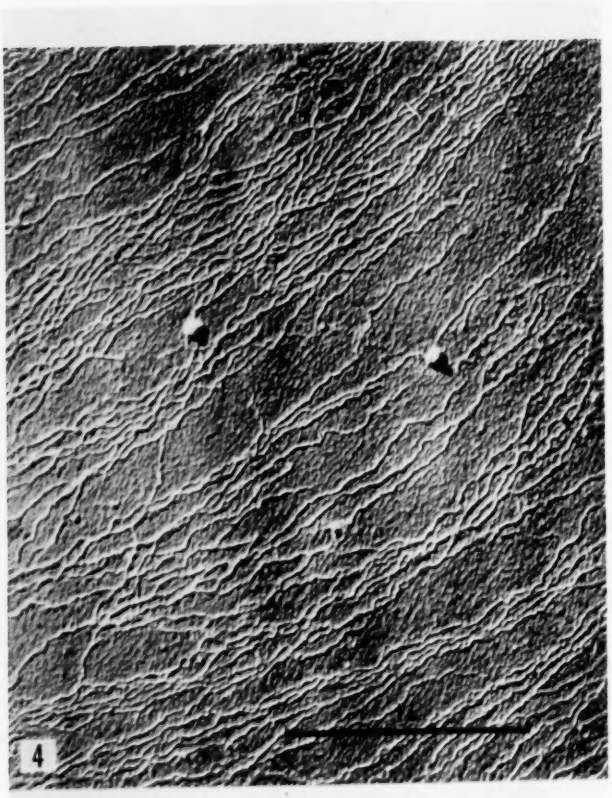

Fig. 4. Electron micrograph of a thymus nucleoprotein preparation (KAHLER and LLOYD[142])
The black line represents the length of 1 μ

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The arrangement is based on and suggests a reason for the approximate equality of these groups which is described above. WATSON and CRICK

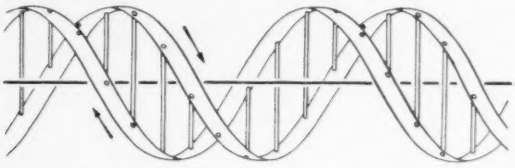
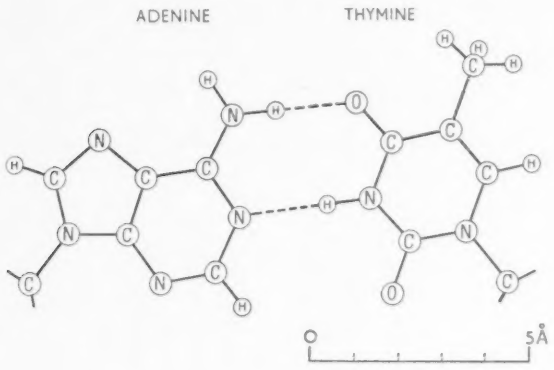


Fig. 3*a*. Suggested structure of deoxyribonucleic acid (WATSON and CRICK[139]). This figure is purely diagrammatic. The two ribbons symbolize the two phosphate-sugar chains and the vertical rods the pairs of bases holding the chains together. The horizontal line is the fibre axis.



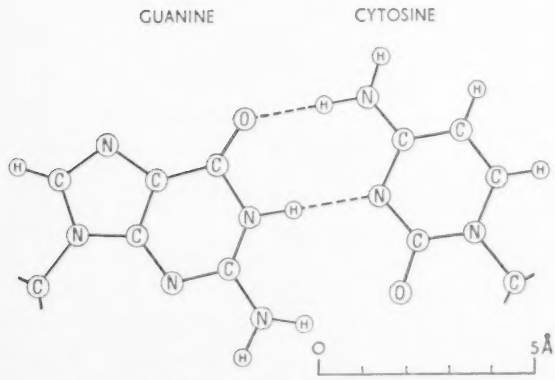


Fig. 3*b*. Hydrogen bonding of purine and pyrimidine bases as suggested by WATSON and CRICK.

find that it is only possible to build up a regular structure in this manner when the bases are combined together in pairs in this way.

The other authors mentioned discuss the X-ray fibre diagrams of

DNA and suggest that the basic requirements suggested by the x-ray data are a helical structure with axis parallel to the fibre length having a pitch of 34 A and diameter of 20 A. This is satisfied by intertwined helical polynucleotide chains with 10 phosphate groups on each helix. The phosphate groups are probably outside and the nitrogeneous basis inside the helix. It is a remarkable fact that very similar patterns have been obtained both from isolated DNA from different sources (calf and pig thymus, wheat germ, herring sperm, human tissue and T 2 bacteriophage; and also from nucleoprotein preparations both intact and reconstituted. It would appear that this structure is not an artefact, but exists also in intact biological systems, such as sperm heads (WILKINS and RANDALL,[140]) and in bacteriophage and transforming principle (WILKINS, STOKES and WILSON[137]). As it is found in DNA from different species, the structure is independent of the varying ratios of the bases. RILEY and ARNDT[150], however, believe that the x-ray scattering curves of nucleoproteins are incompatible with the protein being present in an unfolded form, which this hypothesis would require. The nucleoprotein behaves much more like a gross additive complex than a closely integrated structure.

It is suggested that the groups of polynucleotide chains, when parallel may link sideways in different ways to give the crystalline fibres and semi- or para-crystalline fibres observed under different conditions. In the nucleoprotein it appears to be possible to intertwine the protein between the turns of the nucleotide helix without increasing the separation of the polynucleotide units very much.

Electron micrographs of DNA have been obtained by WILLIAMS[141] and by KAHLER and LLOYD,[142] which show elongated fibres of 15 A diameter, together with globules similar to those described by BAILEY.[143] KAHLER and LLOYD also give a picture of calf thymus nucleoprotein showing long kinked fibres of approximate diameter 24 A (see Fig. 4). LIQUIER-MILWARD[144] has however given pictures showing threads of diameter 100–160 A.

A number of recent studies of the molecular weight and dimensions of DNA by light scattering observations have been made. KATZ[145] determined the scattering envelopes of DNA solutions and found it to be intermediate between the theoretical requirement of rods and coils. He also studied the aggregation caused by the presence of mercuric ions. DOTY and BUNCE[145] studied three samples of molecular weights, 4·0, 6·7 and $4{\cdot}0 \times 10^6$. The sample with the highest molecular weight appeared to exist in 0·2 M sodium chloride as a cylinder 6000 A long and 1500 A in diameter—a relatively small degree of dissymmetry—suggesting a branched polynucleotide chain, rather than a rod or random coil. ROWEN[147] has arrived at a similar conclusions and has suggested that the addition of salts causes a decrease in the effective

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length of the particle. It must be remembered however that light scattering measurements are made at concentrations (c 10^{-4} g/ml), at which a considerable amount of particle interaction may occur in water and the results probably refer to an aggregate. POUYET[148] showed that the reduced viscosity of DNA decreases greatly in the region of 10^{-5} g/ml and approaches the value which it has in salt solutions. This implies that in the limit at very low concentrations the addition of salts does not affect the molecular size or shape, or only affects it slightly. The rapid rise of the reduced viscosity in water at higher concentrations must be due to particle interactions. CONWAY and BUTLER[149] have shown that in this region even very small concentrations of added salts have a marked effect on the viscosity and for this reason it is probable that the forces causing interaction are electrostatic in nature. At any rate, it is clear that except in the case of extremely small concentrations of DNA, the addition of salts may cause a decrease in particle interaction and that the effects of salts cannot be ascribed solely to changes of shape as has been done. It may therefore be necessary to re-interpret much of the data obtained at concentrations of the order of 10^{-4} g/ml.

Studies of the "rigidity" of DNA solutions have been made by FERRY and KATZ[217]; and new interpretations of the dielectric measurements by G. and I. JUNGNER[129].

IV. Role of Deoxyribonucleic Acid and Nucleoproteins

It is impossible in an article of this kind to discuss this question from every angle, but it may be useful to mention some recent speculations and experiments on the functions of nucleic acid and its complexes in the cell nucleus, as they have some influence on the direction and objectives of physico-chemical researches in this field. That the chromosomes carry the genes is a well-established concept in genetics and cytology and it is therefore natural to identify the gene material with their main constituent, viz. the nucleoprotein. The work of BEADLE and TATUM on the hereditary characters in *Neurospora* has shown that in these organisms the ability to form specific enzymes is a unitary hereditary character and therefore controlled in each case by one gene. It was therefore suggested that the function of the genic material in general, and nucleoprotein in particular, to provide a set of patterns or templates, each of which controlled the synthesis of a specific protein or enzyme.[165] Since every protein produced by an organism is not only specific in function, but also, as immunological tests show, specific to the nature of the organism, a very high degree of specificity of the protein manufacturing mechanism is required. It has been difficult to see how the known structure of deoxyribonucleic acid could be capable of providing a sufficient number of variable factors to account for the

enormous number of variations which must occur in the proteins. CALDWELL and HINSHELWOOD[166] suggested that the difficulty could be overcome by supposing that two elements of the DNA structure are implicated in determining the specificity of the residue at each position in a peptide chain. Taking 5 distinct kinds of groups (4 bases and 1 sugar), the number of distinct ways these could be combined in pairs (24) corresponds approximately to the number of material amino acids from which the proteins are constricted. A similar suggestion has been made by DOUNCE[178] (see below); but he permits the specificity at any point in the DNA chain to be determined by the base at this point and by the two neighbours on either side. This gives a greater number of possibilities (4^3) of specificity at this point, but when one point has been determined in this way the number of possible ways of filling the next are rather restricted.

Suggestions of this nature are obviously rather artificial, and an alternative theory which avoids the necessity of any specificity being carried by the DNA has been proposed by HAUROWITZ.[167] In this the DNA is reduced to the role of keeping the peptide chain in an extended state in which it is copied, by a second chain having the same arrangement of amino-acids being laid down adjacent to it, through the operation of the ordinary van der Waals forces such as cause the crystallization of one molecular species out of a solution containing a mixture. It will be recalled that in FURBERG's zig-zag arrangement of the phosphate groups in DNA there are two suitably placed rows of phosphate groups, and it would be quite possible for one peptide chain to be "anchored" on one row, while its copy was being formed on the other. It is also possible that if, as suggested above, the DNA occurs in complex configurations it could control the folding of the peptide chains once formed in an extended state. AMBROSE[169] has suggested that the peptide chains might be formed in the α-configuration and that a change to the β-configuration would enable them to "peel away" from the DNA template.

Theories of this kind are obviously very speculative and lack experimental support at the present time. Many proteins do not possess a large number of basic groups so that they could not be expected to be attached to DNA at more than a few points. The proteins actually found to be associated with DNA are also of rather specialized types, e.g. protamines and histones, for which no function has been discovered. It is conceivable that they are the basic material from which the final proteins are elaborated and that they have sufficient specificity to determine the nature of the finished protein. But there seems no good reason for not admitting that these specialized protein materials may not themselves be a necessary part of the gene and contribute to determining the required specificity. It will be remembered in this connection

that RAUEN, FELIX and co-workers have established the heterogeneity of the protamines of fish sperm. They have shown that eggs of *Salvio fontinalis* can be fertilized by well-washed nuclei of the sperm cells which contain little but nucleoprotein.[170] From the diversity of protamines they believe them to be components of the genes.

There are no strong reasons for thinking that the chromosomes in the germinal cells are identical, or similar in function to those of somatic tissues at any rate on higher animals. The proteins of the latter (histones) differ considerably from those of the germinal cells, and may have quite different functions. There is evidence that protein synthesis occurs in the cytoplasmic particles[182] but at present there is little direct evidence that the chromosomes of somatic tissues are able to manufacture specific types of proteins, such as enzymes.

There is however evidence that in the transforming principles, first discovered by AVERY, MACLEOD and MCCARTY,[171] we have substances capable of bringing about inheritable changes in bacteria which consist of DNA alone. In the purest preparations HOTCHKISS states that the protein has been reduced to 0·02%;[172] the activity is also destroyed by deoxyribonuclease and not by proteolytic enzymes.[173] Denaturing agents, like urea, and also nitrogen mustards destroy the activity.[174]

At least 16 other transforming principles, with similar properties have also been found;[172] and it has also been shown that when DNA is extracted from strains of bacteria which have become penicillin resistant, it is capable of conferring permanent penicillin resistance to sensitive strains inoculated with it.[175]

Although these are all convincing demonstrations that DNA is capable of carrying qualities which, once transmitted, are inherited by the cell descendants, yet they do not necessarily prove that all genes are composed of DNA solely; and it may well be that these particular genes are not concerned with protein synthesis, e.g. the transforming principle which converts the *R* strain pneumococus to the encapsulated type III variety, may only be capable of transmitting the ability to synthesize a certain type of carbohydrate.

Any theory of the mechanism of reproduction must account in the first place for the reproduction of the genes. A possible mechanism of the reproduction of DNA fibres has been suggested by DOUNCE.[178] The first step is the diphosphorylation of the phosphate groups of the nucleotide chain by ATP, probably through the agency of suitable enzymes. This is followed by the attachment of the sugar and base, the specificity of the latter being determined by the basic groups already present on the prototype. No evidence of synthesis of DNA in this way is however available at present and it might equally well occur in the reverse order i.e. by each of the existing bases attracting a complementary base to it, followed by the addition of sugar and phosphorylation.

All such processes must remain speculative until they can be conducted under controlled conditions in the test tube. It may however be noted that STERN has claimed that thymus nucleoprotein, prepared with the aid of low-ionic strength solvents exhibits marked adenosine triphosphatase activity.[179] However ALFREY, STERN, MIRSKY and SACTRON[180] found no ATPase activity in the nucleus.

WATSON and CRICK[164] have pointed out that their proposed structure provides an easy and certain means of reproducing the DNA chain. Since the bases are combined in complementary pairs (adenine with thymine and guanine with cytosine or methyl-cytosine), each half of the double helix is completely complementary to the other half. If the two halves are separated, either spontaneously or by an enzyme action, each will be able to add its complementary half in a completely unambiguous way, so that the result will be two complete DNA fibres in place of and identical with the original one. This is the first convincing demonstration of how a specific arrangement of groups in a biological structure could reduplicate itself. The number of ways of arranging the bases in a double helix of this kind is exactly the same as in a single polynucleotide chain, i.e. the amount of variation possible from point to point is comparatively small and apparently insufficient to determine the specificity of the amino-acids in a peptide chain.

This model is also capable of accounting for the "crossing-over" of segments of the chromatids, which occurs during meiosis, if it is supposed that at a point of contact of two nucleotide "half-chains" a newly synthesized fibre which is being formed along one of them may continue along the other from the point of contact. In order that crossing-over may occur at the same point in the two homologous chromatids (see p. 198) it is obviously necessary for contacts to occur at equivalent loci in the two "half-chains." It may be that the nucleotide pattern is designed to secure this exact alignment for crossing-over.

The finding of BINKLEY and OLSON[181] that the dipeptide splitting enzyme of pig kidney and other tissues is a pentosepolynucleotide may be mentioned here, as it points to the possibility of other enzymic, or at least catalytic properties, of nucleic acids.

V. SOME REACTIONS OF THE NUCLEOPROTEIN COMPLEX

A. *Action of chemical agents on DNA*

Very little work has been done, owing to its complexity, on the action of reagents on the intact nucleoprotein. Owing to its suspected role as the bearer of the genes, a considerable amount of work has appeared on the action of various chemical agents on DNA, especially of substances which have marked mutagenic and carcinogenic properties and of ionizing radiations. In the first place we shall describe the effects

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of some simpler agents, such as acids and alkalies and denaturing agents like urea. The fact that on acid or alkaline treatment the viscosity of DNA solutions falls to comparatively low values was noted by CREETH, GULLAND and JORDAN[185] who found that recovery to the original values took place when the solutions were made neutral again. CHARGAFF and ZAMENHOF found that the viscosity of thymus nucleic acid began to diminish even at *p*H 5·8 and that on bringing such solutions to neutrality again they were markedly thixotropic and also differed in thermal stability. They interpreted this as due to a kind of denaturation of the original DNA by acids.

GULLAND, JORDAN and TAYLOR had found that after exposure to acids and alkalies the titration curve of DNA was modified in such a way that certain groups not initially titratable in the *p*H ranges 5–6 and 10–12 became so. They interpreted this to mean that some of the hydroxyl and amino or imino groups (—NH_2 and >NH) of adjacent bases were hydrogen bonded with each other and were not titrated until sufficiently high or low *p*H's were reached which were able to break these bonds and so render the groups accessible to titration.

This anomalous titration behaviour has also been studied by COSGROVE and JORDAN,[189] LEA and PEACOCKE[190] and by SHACK and THOMPSETT[191] who found that treatment with both acids and alkalies had permanent effects on the titration curves of DNA.

HORN, LEVAY, POUYET and SADRON[192] have also studied the effect of changes of *p*H on the properties of thymonucleic acid. At *p*H 3·8 (in molar sodium chloride) the intrinsic viscosity is considerably lower than at *p*H 7, and does not return to the original value when the solution is re-neutralized. Light scattering, streaming birefringence and extinction angle measurements however indicate that the particle is *more* aniso-diametric in the neutral solutions.

CAVALIERI, KERR and ANGELOS[193] also found that between *p*H 8 and 12 fewer equivalents of alkali are required for titration than are predicted by the known proportions of the bases and suggested a linkage between a phosphoryl group and the —NH—CO— groups.

That acids and dilute alkalies bring about permanent changes in the state of DNA has also been indicated by the changes in sedimentation homogeneity.[194] REICHMANN, BUNCE and DOTY[195] studied the light scattering of DNA solutions between *p*H 6·5 and 2·6 (in 0·2 M NaCl). The molecular weight remained unchanged but the results indicated a substantial contraction of the molecule at *p*H 2·6, which was substantially reversed on returning to the original *p*H. They suggested that very little irreversible degradation occurs at *p*H's above 2·6, and they suggest that the discrepancy between this behaviour and that observed by others is due to their method of gradually changing the acidity by dialysis against the new buffer solution.

The fact that urea and other protein denaturants reduce the viscosity of DNA solutions was noted by JENRETTE and GREENSTEIN.[196] The effect of this agent and also of phenol has been studied in some detail by CONWAY and BUTLER.[183] They find that the effect is largely irreversible i.e., the viscosity does not return to its original value on removing the agent by dialysis. The character of the viscosity is also greatly changed in that the shear dependence is greatly diminished. The molecular weight was reduced to about half and it was suggested that the effect of these agents is due to their ability to break the hydrogen bonds which maintain the nucleic acid in its original configuration and so allow it to assume a more compact configuration. Effects of this kind are brought about by comparative large concentrations of denaturing agent, but it was shown that small concentrations brought about similar effects over a long period of time.

The effect of various mutagenic and carcinogenic agents on desoxyribonucleic acid has been extensively studied, especially substances of the "mustard" class which have been shown to be highly effective as mutagenic[197] and carcinogenic agents[198] and in inactivating animal viruses (HERRIOTT).[199] CHANUTIN and GJESSING[200] had observed limited changes of viscosity produced by the nitrogen mustards, di(chlorethyl) methylamine $CH_3 . N(CH_2 . CH_2 . Cl)_2$ and tri-chlorethylamine $N(CH_2 . CH_2 . Cl)_3$. BUTLER and SMITH[201] showed that in the reaction of the former an almost complete loss of viscosity of the DNA solution occurs. A number of studies have been made of the chemical and physical changes brought about by these and similar reagents (e.g. "mustard gas" $S(CH_2 . CH_2 . Cl)_2$). The chemical studies of their reactivity by HANBY and RYDON[202], GOLUMBIC, FRUTON and BERGMANN,[203] STEIN, MOORE and BERGMANN,[204] and ROSS[205] established their character as alkylating agents capable of reacting with hydroxyl, amino and carboxyl-groups. ELMORE, GULLAND, JORDAN and TAYLOR[206] studied the reaction products of the sulphur mustard with DNA and showed, mainly by titration data, that reaction with the amino and hydroxyl groups of the bases occurred and also with the primary (non-terminal) phosphate groups. Similar reactions were found to occur with the di(chlorethyl)methylamine by PRESS and BUTLER.[207] Physicochemical studies of the "degradation products" of these agents on DNA have been made by CONWAY, GILBERT and BUTLER[208] and by BUTLER, GILBERT, JAMES and ROSS.[209] It was found that the change of viscosity in the initial period of the reaction is not accompanied by any large changes of molecular weight and must be due to either changes of shape or of mutual interaction of the DNA particles. That the loss of viscosity was due to a simple coiling up of the nucleate particles, when the phosphate groups were partly neutralized by reaction with the alkylating agent, was shown to be improbable as the

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change of charge carried by the particles is not sufficiently large. The complete alkylation of the amino groups of the bases will necessarily break any hydrogen bonds in which they are implicated; and it is obvious that reagents of this type will be capable of breaking down the initial configuration of the DNA. But much of the initial decrease of viscosity may be due to the diminution of the interaction of the particles, which is very sensitive to the partial neutralization of their charge, which decreases their polyelectrolyte character.

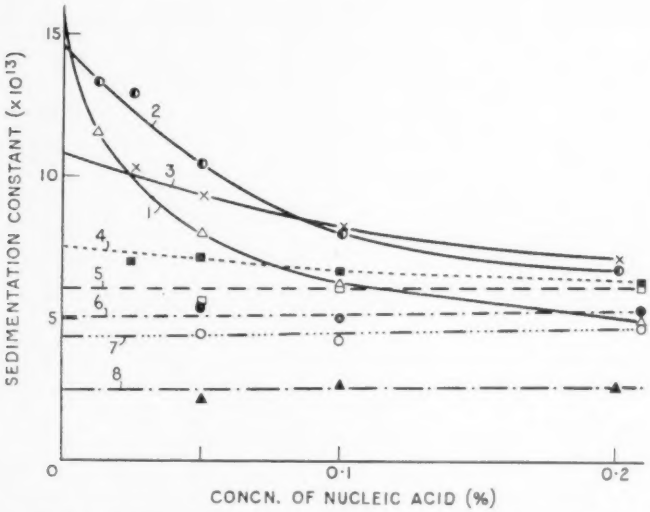


Fig. 5. Sedimentation constants as function of nucleic acid concentration after treatment with the nitrogen mustard HN2, for various times in sodium hydrogen carbonate. Reaction carried out with nucleate concentration 0·2% and amine concentration 0·2%. (1) Original nucleic acid. (2) After 1 day. (3) After 3 days. (4) After 5 days. (5) After 7 days. (6) After 7 days + 7 days' dialysis at 0°. (7) After 18 days, no dialysis. (8) After 7 days + 7 days' dialysis. (BUTLER, GILBERT and JAMES[209])

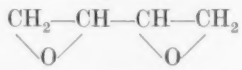
After the initial loss of the characteristic viscosity further changes take place, which are shown by the slow decrease of the sedimentation constant (Fig. 5), which continues for 14 days or more. This behaviour and molecular weight determinations of the final state, which give values between 10^4 and 10^5, indicates that a gradual decrease of molecular weight occurs on standing. It has been suggested that this slow breakdown of the DNA particle is due to the hydrolysis of the tri-substituted phosphate formed when the doubly esterified phosphate esters react with the "mustard" residues. Evidence of the greater ease of hydrolysis of tri-phosphate esters has been given by Ross.[210]

A decrease of the viscosity of DNA solutions is also brought about

by the presence of other substances which have been shown to possess mutagenic or carcinogenic properties e.g.

ethylene-imine

di-epoxybutane



trimethylol melamine; 2 : 4 : 6-tri-ethylene imino-1 : 3 : 5 triazine.[211] The actions have not been investigated in detail and it cannot be claimed that this kind of behaviour is characteristic of mutagenicity or carcinogenicity since similar effects are produced at low concentrations by hydrogen bond breaking agents such as urea and phenol.

In the case of the "mustards" difunctionality is not a necessary factor either for biological activity or for decreasing the viscosity of DNA. The monofunctional compound $(CH_3)_2N \cdot CH_2CH_2Cl$ has been shown by KÖLMARK and WESTERGAARD[212] to be nearly as active in producing mutations of neurospora as $CH_3 \cdot N(CH_2CH_2Cl)_2$ and it also causes the loss of the viscosity of DNA; the most noticeable difference between the two compounds being a lag or induction period in the case of the former compound before the viscosity begins to fall.[213] This may be due in part to the slowness of the reaction and the fact that it must proceed to a certain extent before the breakdown of the TNA structure begins.

Many polycyclic compounds without polar groups appear to combine with DNA. They are only very slightly soluble in water and the solubility usually decreases with increase of molecular size. It was shown by WEIL-MALHERBE[214] that the solubility is greatly increased in the presence of caffeine and other purines. The increase is probably due to the formation of molecular complexes, but a large molecular ratio of purine to hydrocarbon is required to produce solubilization. BOOTH and BOYLAND[215] have found that DNA, probably owing to its purine content, also solubilizes polycyclic hydrocarbons, as well as other substances of a known carcinogen nature, e.g. benzcarbazoles and benzacridines. Small changes of ultraviolet and infrared absorption spectra of the compounds are observed.[216] These effects are not however confined to carcinogenic compounds, so that it is not certain if they play any part in carcinogenesis.

B. *Effects of Radiations on Nucleic Acids and Nucleoproteins*

1. *Ionizing radiations*

It is now widely accepted that the effects of ionizing radiations in dilute aqueous solutions are to be attributed mainly to the indirect

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action of H and OH radicals[220] formed from the water by the absorption of energy in the liquid. Subsequent reactions between radicals and with water can lead to the production of hydrogen peroxide and oxygen. In the presence of oxygen other reactions are possible in which the HO_2 radical and H_2O_2 molecules are formed in greater quantities.[221]

Primary effects on nucleic acids—The effect of x-rays on nucleic acid must be studied by observations of changes in some characteristic property of the material; in most work this has been the high non-Newtonian viscosity or the birefringence. When a biological property, e.g., the transforming activity of some nucleic acids is available, it might be used with greater advantage. SPARROW and ROSENFELD[225] showed that the loss of viscosity and birefringence of deoxyribonucleic acid was an exponential function of the radiation dose. This observation has been confirmed by several workers;[222], [226], [231] the dependence of loss of viscosity or birefringence on dosage appears to become linear with dose at low doses as found by TAYLOR, GREENSTEIN and HOLLAENDER[223] and by G. C. BUTLER.[231] Changes in the sedimentation constant, (S), of deoxyribonucleic acid after x-irradiation have been examined by several workers[223], [237], [240] but the results are rather discordant. The discrepancies may arise from the varying conditions, e.g., concentrations of salts or of DNA, used in these determinations; furthermore it is not always clear if extrapolation of S to zero concentration was attempted. CONWAY, J. A. V. BUTLER and GILBERT[240] measured both the sedimentation constants and the diffusion constants of x-irradiated DNA at various concentrations and found after radiation with 40,000 r an approximate molecular weight decrease of the DNA from $1{\cdot}5 \times 10^6$ to $5{\cdot}3 \times 10^5$.

Since the physical effects discussed above are observed in dilute aqueous solutions of DNA, they are to be regarded as *indirect* effects of the free radicals generated from the water. Experimental evidence for this view was obtained by G. C. BUTLER[231], [232] who, following LEA's[243] criteria for indirect action of ionizing radiations, showed that the loss of viscosity was inhibited when glucose, methanol or degraded DNA were present as protective agents. Similar results were found by CONWAY[229] using cysteinamine, cystinamine and NaCN. From the protective action of the degraded DNA it was inferred that DNA degraded to a non-viscous material reacted with radicals to an extent equal to that of the undegraded nucleic acid. It was also shown by G. C. BUTLER as well as by STERN, BRASCH and HUBER[242] and LIMPEROS and MOSHER[237] that irradiation of DNA in the *solid* state or in *frozen* aqueous solution produced little degradation as determined by viscosity measurements, thus demonstrating that mobile free radicals which are produced in liquid aqueous solution are necessary for the

degradation to take place. Further confirmation of the indirect nature of the degradation was obtained by LIMPEROS and MOSHER[237] who showed that *no* degradation took place when DNA was irradiated in ethylene glycol solutions. The generation of radicals (mainly OH) from hydrogen peroxide by U.V. irradiation or by reaction with ferrous ions, has been shown by J. A. V. BUTLER and SMITH, J. A. V. BUTLER and CONWAY,[241] SCHOLES and WEISS[234], [235] and LIMPEROS and MOSHER[18] to bring about both physical and chemical changes in DNA which closely simulate those produced by ionizing radiations, thus supporting the indirect action of the latter. A protective action of cysteinamine, cystinamine and NaCN is also observed in these reactions.[229]

Although the radiation chemistry of oxygen-containing water is rather different[221] from that of deoxygenated water it is of interest that several workers viz. G. C. BUTLER[231], D. B. SMITH and G. C. BUTLER,[232] J. A. V. BUTLER and CONWAY[226] and LIMPEROS and MOSHER,[237] are in agreement that the *primary* effect of X-rays on the viscosity (and birefringence) of DNA is similar whether oxygen is present or completely absent in the solution. However, in the presence of oxygen, marked post-irradiation effects are observed[222], [226], [227], [239] which will be discussed below.

A considerable amount of work has been done on the chemical changes caused by radiations on nucleic acid and its constituents. SCHOLES and WEISS[234], [235] detected the formation of both ammonia and inorganic phosphate with doses of ca 30,000 *r*, from pentose nucleic acid, highly polymerized DNA, and ammonia from adenine. The liberation of ammonia under these conditions is enhanced by the presence of oxygen and inhibited by hydrogen; conversely the liberation of inorganic phosphate is enhanced in the presence of hydrogen. These results suggest that the deamination process is brought about mainly by an oxidative mechanism whilst the splitting of the phosphate ester groupings and the subsequent liberation of PO_4''' is at least partly brought about either directly or indirectly by hydrogen radicals.

With larger radiation doses correspondingly greater chemical effects are observed. With doses in the range $1 - 4 \times 10^6$ *r*. SCHOLES and WEISS[236] have shown that in addition to depolymerization and the liberation of ammonia and inorganic phosphate, ring fission of constituent purine and pyrimidine bases occurs giving products of oxidation and hydrolysis such as urea and oxalic acid. Free purine and pyrimidine bases are also liberated, glycosidic linkages are broken and the sugar moieties are oxidized to carboxylic acids and an overall increase in titratable acid groups is observed. Similar chemical effects have been observed on treatment of the nucleic acids with hydrogen peroxide and ferrous ions.[236] J. A. V. BUTLER and CONWAY[241]

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studied the action of radicals formed from hydrogen peroxide by U.V. irradiation in the presence of DNA. The effects are quite similar to those of x-rays, both qualitatively and quantitatively on the assumption that two hydroxyl radicals have been formed for each hydrogen peroxide molecule decomposed. Among the effects observed are fission of sugar-phosphate links with liberation of free phosphate, oxidation of the sugars, decrease of the purine-bound deoxyribose and decrease of the extinction in the U.V. at 2600 Å (and proximate region) indicating destruction of the absorbing ring systems of the bases, and deamination.

STERN *et al.*[242] using bursts of high energy electrons and TAYLOR *et al.*[223] using x-rays, found that up to 10,000–20,000 *r* the ultraviolet absorption spectrum remained unchanged, although the viscosity of the DNA was considerably diminished by this treatment. The viscosity of the nucleic acid is therefore extremely sensitive to small chemical changes occurring in the molecule since at these dosages TAYLOR *et al.*[223] were unable to detect any breakdown products e.g. dialysable NH_3, PO_4''' or organic phosphate (cf. SCHOLES and WEISS[233], [234]).

More detailed information about the chemical nature of the mechanism of these overall chemical effects can be obtained by the study of radiation effects on isolated constituents and analogues of constituents of nucleic acids.

The liberation of both ammonia and inorganic phosphate from RNA[235] (and probably DNA) is *p*H dependent in a complex but similar way, each curve showing a minimum in the region of *p*H 5 and a maximum at *p*H 8. Similar effects occur with adenylic acid. The liberation of ammonia from adenine, however, shows a continuous fall with increasing *p*H from *p*H 1 to *p*H 12, indicating that the presence of an ionized phosphoryl group in adenylic acid modifies considerably the extent of ammonia formation, particularly at higher *p*H's.

Radiation effects on purines and pyrimidines themselves parallel the overall effects observed when nucleic acids are irradiated. In general with compounds containing amino groups, a much greater liberation of ammonia occurs than with others, although some ammonia arises from destruction of the heterocyclic rings.[236] Oxalic acid, urea, hydroxylamine and guanidine have also been detected. It appears that the pyrimidine bases are much more susceptible to attack by the radicals than the purines; thus, both J. A. V. BUTLER and CONWAY[241] comparing the effects of OH radicals on adenine and thymine and SCHOLES and WEISS[236] comparing adenine and uracil, found relatively much greater destruction of the pyrimidine than the adenine. This is paralleled by the effects of U.V. on pyrimidines and purines[244] and the

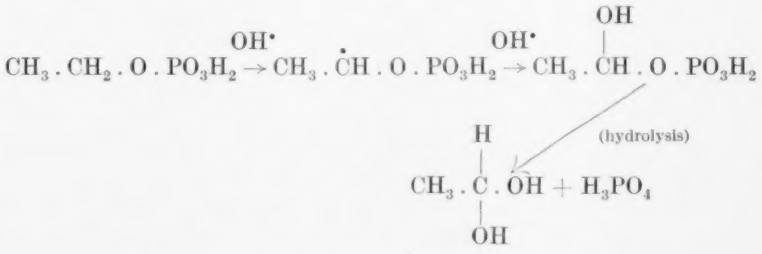
effect appears to arise on account of the greater reactivity of the more ethylenic[258] 4–5 bond in pyrimidines. This preferential attack on the pyrimidines may also occur in the irradiation of the nucleic acids though this has not hitherto been experimentally demonstrated.

The mechanism of phosphate liberation from nucleic acid and other phosphate esters e.g., mono, di- and tri-ethyl phosphates, has been studied in some detail by J. A. V. Butler and Conway,[241] using oxidizing radicals (OH and HO_2) from hydrogen peroxide.

The results with the di- and tri-esters can be accounted for on the assumption that the liberation of phosphate occurs as the result of two and three consecutive reactions respectively. If it is assumed that the chance of any phosphate-ester link being broken is independent of the state of the others, it is found that the rate of liberation of phosphate from all three compounds can be accounted for in terms of the single constant which represents the number of phosphate-ester bonds broken by unit dose of radicals.

This treatment has been extended to the liberation of phosphate from DNA solutions. On the assumption that the amount of terminal (singly bound) phosphate is negligible it is found that the amount of phosphate liberated should be proportional to the square of the radiation dose. This has been found to be reasonably well satisfied both with photochemically generated radicals and with x-rays. However, the first 1% of the phosphate liberated appears to be less firmly bound than the remainder, although the amount is still proportional to the square of the dose.

The breakage of phosphate-ester bonds is however not the result of a direct action of the radicals. In the case of the ethyl phosphates it was found that an oxidation of the ethyl groups to aldehyde and acetic acid was always equivalent to the amount of phosphate liberated. It was inferred from this that the liberation of phosphate occurs as a consequence of the oxidation of the alkyl groups, as in the scheme:



$$CH_3 . CH_2 . O . PO_3H_2 \xrightarrow{OH^\bullet} CH_3 . \dot{C}H . O . PO_3H_2 \xrightarrow{OH^\bullet} CH_3 . CH(OH) . O . PO_3H_2$$

$$CH_3 . CH(H)(OH) + H_3PO_4 \quad \text{(hydrolysis)}$$

The efficiency of the liberation of phosphate by photochemically generated hydroxyl radicals is however very high and with small doses approaches two phosphates for each hydrogen peroxide molecule

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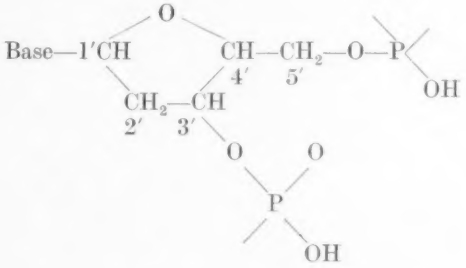
decomposed. This suggests that after the initial step, the process is completed by a short reaction chain involving water e.g.,

$$CH_3 . \dot{C}H . O . PO_3H \xrightarrow{H_2O} CH_3 . \underset{\displaystyle OH}{\underset{|}{C}}H . O . PO_3H + H^{\bullet},$$

$$CH_3 . CH_2 . O . PO_3H + H^{\bullet} \rightarrow CH_3 . \dot{C}H . O . PO_3H + H_2, \text{etc.}$$

In the same way the liberation of phosphate from nucleic acids is probably a consequence of the oxidation of the sugar groups. SCHOLES and WEISS have found[235a] in experiments on glycerophosphates that, after irradiation, the amount of phosphate liberated by acids is increased and suggest that an oxidized compound is formed in which the phosphate is "acid-labile." They found a similar increase of "acid-labile" phosphate in the irradiation of DNA, which is attributed mainly to the oxidation of the C4′ carbon of the deoxyribose moiety. They suggest that oxidation at C3′ or C5′ must lead to an immediate breakage of the nucleotide chain:



Radiation after-effects on nucleic acids—The existence of various types of after-effect of radiations in living cells[246] makes the study of after-effects with nucleic acid *in vitro* one of immediate importance. TAYLOR, GREENSTEIN and HOLLAENDER[223] first demonstrated an after-effect of x-irradiation with DNA and showed that in addition to a lowering of the characteristic non-Newtonian viscosity during irradiation, the viscosity continued to fall for many hours after the end of the irradiation. With moderate doses of radiation, e.g., 10,000–20,000 *r*, the after-effect on the viscosity is considerably greater than the primary effect which suggests that the after-effect may be the process of greater importance biologically. Similar effects were subsequently observed by GRINNAN and MOSHER[239] with RNA from rat liver. J. A. V. BUTLER and CONWAY[226] showed that the after-effect with DNA was largely dependent on the presence of oxygen during the irradiation; if the irradiation was carried out in nitrogen little change of viscosity occurred after the first hour or two; it was also

found that the primary effect of the irradiation was much the same whether oxygen was present or not, as has been found by other workers.[231], [232], [237] These results suggested that the after-effect must be due to a product of the primary radicals and oxygen. It is well known that hydrogen peroxide is formed under these conditions in small quantities but TAYLOR, GREENSTEIN and HOLLAENDER[223] and later LIMPEROS and MOSHER[237] were unable to demonstrate any effect of hydrogen peroxide on DNA up to a concentration of 10^{-2} M H_2O_2 so that it appeared that H_2O_2 was not the agent responsible for the after-effect. However, BUTLER and CONWAY[226] found that most of their preparations of highly polymerized DNA were spontaneously susceptible to the action of H_2O_2 down to a concentration of 10^{-4} M. By determining the concentration of H_2O_2 formed during x-irradiation of oxygenated DNA solutions, they showed that although significant quantities of H_2O_2 were formed, they were not quantitatively sufficient to produce the correct magnitude of the after-effect observed in x-irradiation, when these concentrations of H_2O_2 from a bottle were used. CONWAY and BUTLER[227] and CONWAY[229] later showed that some samples of DNA were completely insensitive to H_2O_2 whilst others were in varying degree, reactive. Insensitive samples could be made sensitive by addition of traces (10^{-5} M) of activating materials, e.g., Fe^{++}, ascorbic acid, so it therefore appears that the spontaneous sensitivity towards H_2O_2 depends on traces of impurities or degradation products introduced during the preparation of the DNA.

That the after-effect is not entirely due to H_2O_2 alone is further supported by several experimental facts: the after-effect can still be detected after an irradiated DNA solution is freeze-dried to remove all volatile compounds, e.g., H_2O and H_2O_2, and reconstituted to the same volume with pure water; samples which are insensitive to H_2O_2 still show a marked radiation after-effect. This latter behaviour could be explained if the radiation-damaged DNA became sensitive to H_2O_2 by formation of reactive groupings during the irradiation. This was found to be the case experimentally,[229], [230] although the action of H_2O_2 at an appropriate concentration on radiation damaged DNA was still not sufficient to simulate quantitatively the magnitude of the after-effect.

These experimental facts lead to the view that although the action of H_2O_2 can partially account for the after-effect some other process must also occur. J. A. V. BUTLER and CONWAY[226], [229], [230] suggested that the radicals HO_2, which are formed secondarily in oxygen solutions by the processess

$$H_2O \longrightarrow OH \text{ and } H$$

and

$$H + O_2 \rightarrow HO_2$$

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could directly form unstable nucleic acid peroxides or hydroperoxides which subsequently decompose to products of lower viscosity. It should be noted that HO_2 radicals can also be formed from H_2O_2 by the reaction $H_2O_2 + OH \rightarrow HO_2 + H_2O$. Alternatively, nucleic acid radical species may also be formed which could react with molecular oxygen to give peroxidic intermediates.

More recently SCHOLES and WEISS[235], [235a] have suggested that the after-effect is due to slow hydrolytic fission of labile phosphate esters, formed by the oxidation of the C4′ group of the sugar though no experimental demonstration of an increase of free phosphate with time was made.

DANIELS, SCHOLES and WEISS[235b] have found that the "after-effect" is not completely eliminated in solutions irradiated *in vacuo*, although it is reduced to about one third of the value it has in oxygen-containing solutions. The reason for the discrepancy between this finding and that of CONWAY and BUTLER lies in the fact that the latter diluted their solutions (x 2) before measuring the viscosity. The slow change then observed after vacuum irradiation is then no greater than in un-irradiated controls, and is completely eliminated when the solution is diluted with a salt solution. This means that the slow change is due in the main to changes in the interactions of the DNA particles consequent upon their damage by x-rays.[218]

Protective agents, e.g. NaCN, cysteinamine, cystinamine[247] have no inhibiting action on the after-effect when added after the irradiation which is also consistent with the view that the after-effect is mainly an intramolecular effect. (When added before irradiation, both primary and after-effects are inhibited, thus suggesting, as would be expected, that the after-effect is dependent directly or indirectly on the action of the primary radicals.)

Radiation effects on DNA in vivo—Direct studies of radiation effects on nucleic acids or nucleo-protein *in vivo* are difficult and most work (e.g., 248–251) has been confined to biochemical studies of radiation effects on nucleic acid metabolism. However, LIMPEROS and MOSHER[237], [238] examined the physico-chemical properties of thymus DNA isolated from rats "immediately" and 24 hours after x-irradiation by 1000 and 2000 *r*. "Immediately" after irradiation the isolated DNA showed no depolymerization as determined by viscosity and streaming birefringence measurements, although its N/P ratio was decreased from 1·84 (controls) to 1·58 for 1000 *r* and 1·22 for 2000 *r*, indicating splitting off of purine or pyrimidine bases. The ultraviolet extinction was also diminished. Thiourea present at a concentration of 2·6 g/kg body weight showed considerable protective action with respect to the lowering of N/P ratio. When the DNA was isolated after 24 hours, however, there was complete lack of streaming

birefringence and structural viscosity although there was *no* decrease in the N/P ratio. The depolymerization was inhibited to some extent by the presence of thiourea, and was linearly proportional to x-ray dosage between 250 and 1000 *r*; an atmosphere of 10% O_2 + 90% N_2 breathed by the animals caused less depolymerization than when air was used and 100% O_2 caused increased depolymerization in some cases. The degree of depolymerization was related to the mortality amongst the rats.

Although these effects are strikingly similar to those observed *in vitro*, the level of dosage at which they occur is about 10^3 times lower and in fact the chemical quantity of radicals produced by 250–1000 *r* is chemically insufficient, by a similar factor, to produce the chemical degradation implied by a change of N/P from 1·8 to 1·22 unless a chain reaction of considerable length is postulated. This would be unlikely *in vivo* where many reactive compounds are present that could act as chain terminators in a free-radical chain mechanism. It must therefore be concluded that the results of LIMPEROS and MOSHER very probably are not related to those obtained *in vitro* and the great sensitivity of the DNA *in vivo* must be attributed to some interference with metabolic processes controlling DNA synthesis and breakdown.

It is obvious that on any model, such as that of WATSON and CRICK, in which the reproduction of the compound is controlled by the positions of the amino- and hydroxyl groups, any reagent which is capable of reacting with these groups, either by removing them, as in the case of x-rays, or substituting the hydrogens by more massive groups, will interfere with the process of synthesis, and the blockage of the synthetic process at only one point in the nucleotide chain may destroy its continuity and so cause a break in further copies. Chromosome breaks caused by radiations and by carcinogenic chemicals may arise in this way. If chromosomes are formed by a multiplication of a single nucleotide thread, it is possible that the chemical reaction of a single molecule or radical at one point of the original nucleotide, might produce an effect which is large enough to be visible through the microscope. In any case such a mechanism is capable of giving rise to an enormous multiplication factor between the magnitude of the initial reaction and that of the resulting biological action.

Action of ionizing radiations on nucleoproteins—Hitherto little work has been carried out on radiation effects on nucleoproteins of the cell nucleus. SPARROW and ROSENFELD[225] found the viscosity decrease of thymus nucleohistone was exponentially related to x-ray dose in the range 20,000–120,000 *r* as was the case with TNA, but the relative lowering of viscosity at a given dose was less. This may be due to the effective protective action of the histone moiety in the nucleoprotein complex; a similar behaviour was observed by G. C. BUTLER.[231]

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The effects of x-irradiation on chicken erythrocyte nuclei were compared *in vitro* and *in situ* by ERRERA[252] who found evidence for the usual indirect action of the radiation on dilute nucleoprotein gels *in vitro*; however the results, *in situ*, could best be explained by some direct action. This is a point of general importance in arguing by analogy from *in vitro* effects to *in vivo* effects, for the usually greater proportion of solute *in vivo* systems may make direct effects of ionizing radiations of greater relative importance as was pointed out by WEISS[235] (discussion). Experiments at higher concentrations of nucleoproteins and nucleic acids would provide a better basis for comparison.

II. Effects of other radiations on DNA

The effects of ultrasonic radiations, in general, parallel those of ionizing radiations; rapid depolymerization occurs both by sonic (900 c.p.s.,[255]) and ultrasonic (600,000 c.p.s.[254], [257]) radiation and viscosity and streaming birefringence is lost. After long irradiations, e.g., 8 hours. ZBARSKII *et al.*[257] have observed slight chemical changes e.g., ammonia is liberated. The titration curves for the irradiated DNA indicate that considerable chemical changes have taken place in the molecule. Liberation of titratable secondary phosphoryl groups has been found by LEE and PEACOCKE[256] and the characteristic hysteresis in the titration curve, which has been attributed to hydrogen bonding, is much decreased. These results suggest an extensive intramolecular breakdown comparable with the effects observed with ionizing radiations. Chemically, the processes occurring may in fact be to some extent identical since in addition to cavitation and micro-mechanical effects in the solution, free radical formation from the water has been suggested.[259]

The effects of ultraviolet radiation have been reviewed by ERRERA in Vol. III of this series.[253]

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CHROMOSOME BREAKAGE

P. C. Koller

"It is true to say that in scientific borderlands wholly new concepts arise as a result of new intellectual interactions. . . . We must specialise in our methods but we should share our interest."

SIR FREDERICK GOWLAND HOPKINS
Linacre Lecture 1938.

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INTRODUCTION

The chromosomes are important components of the cell because they carry the genes, the physical units of heredity which determine the morphological and behaviour pattern of the organism. The chromosomes are the essential elements of heredity, variation and evolution, and recently light has been thrown also on their role during growth and differentiation.

The chromosomes are transmitted by mitosis during which process they divide and are equally distributed between the daughter cells. Cytoplasmic disturbance follows the loss of a chromosome or chromosome parts which can cause the death not only of the cell but of the entire organism. Their components have the property of self-duplication and if they become altered, they can perpetuate themselves in the altered form. Changes of many kinds occur in the chromosomes, which are either natural events or experimentally induced effects. The study of these phenomena enriched fundamental knowledge and now provides the basis by which the behaviour and organization of the chromosomes may be controlled. The experimentally induced changes are of particular interest. They are the criteria by which the biological efficiency of ionizing radiations is measured, and the impact of chemical agents upon intracellular systems is revealed.

The present article describes various kinds of chromosome breakage. It is necessarily limited in its scope since new facts are emerging daily and new ideas to explain them follow. Emphasis has been placed on the biological aspect of chromosome breakage, based upon selected examples.

THE STRUCTURE OF CHROMOSOMES

The chromosomes can be seen during mitosis. They are composed of polypeptide chains to which deoxyribose nucleic acid is attached, so giving the characteristic Feulgen staining. Their length varies between

0·5 μ and $\frac{1}{4}$ mm. The latter is the length of the giant polytene chromosome in the cells of the salivary glands of *Drosophila*.

The chromosome has a dynamic centre, the *centromere*, the position of which determines the shape of the entire chromosome (Figs. 1–3). The separation and migration of the daughter chromosomes during ana-telophase is directed by the centromere along the spindle fibre towards the opposite poles. It is necessary for the active movement of the chromosomes, since without the centromere the movement is only passive and follows the streaming of the cytoplasm. "*Acentric*" chromosome fragments, whatever their size, are usually left behind the other chromosomes during anaphase and form the *micronuclei* which are scattered in the cytoplasm (Fig. 4).

The position of the centromere is marked by a constriction in the chromosome thread. Beside the primary or centromere constriction the chromosome may have a secondary constriction, which is associated with the formation of the nucleolus during the resting stage. The large M-chromosome of *Vicia* is a classical example; it has a median centromere (hence labelled as the M-chromosome), and a subterminal secondary or nucleolar constriction in one arm of the chromosome. The short segment lying distal to the secondary constriction is the "satellite" (Fig. 5).

The chromosomes persist between two mitoses. During interphase they are enclosed within the nuclear envelope and undergo duplication. When they enter the prophase of mitosis they are composed of two sister *chromatids* which are held together at the centromere. The latter divides only at the end of metaphase. There is not enough experimental data yet to show how the deoxypentose nucleic acid synthesis during interphase is related to the doubling of the chromosome filament.

The completeness of the chromosome set is a pre-requisite for normal functioning of the cell. A deficient nucleus resulting from the loss of acentric fragments, causes the death of the cell.[1]

When the chromosomes of some plants (*Trillium*, *Fritillaria*, *Vicia*, etc.) are exposed to low temperature, particular segments of the metaphase chromosomes fail to give the expected Feulgen staining[2] (Figs. 6, 7). These regions are called *heterochromatic* and are distinguished from the *euchromatic* parts which show normal behaviour. It is likely that these regions differ not only in function but also in molecular organization. Using azur-B, Saez[3] found that the heterochromatic sex chromosome of grasshoppers stained metachromatically, whereas the euchromatin stained orthochromatically.

In the salivary gland of *Drosophila* the heterochromatic sex-chromosome, and the heterochromatic segments of the other chromosomes fuse and form the *chromocentre* (Fig. 8). Such centres are present in the resting nuclei of plants and animals, including man, and give a

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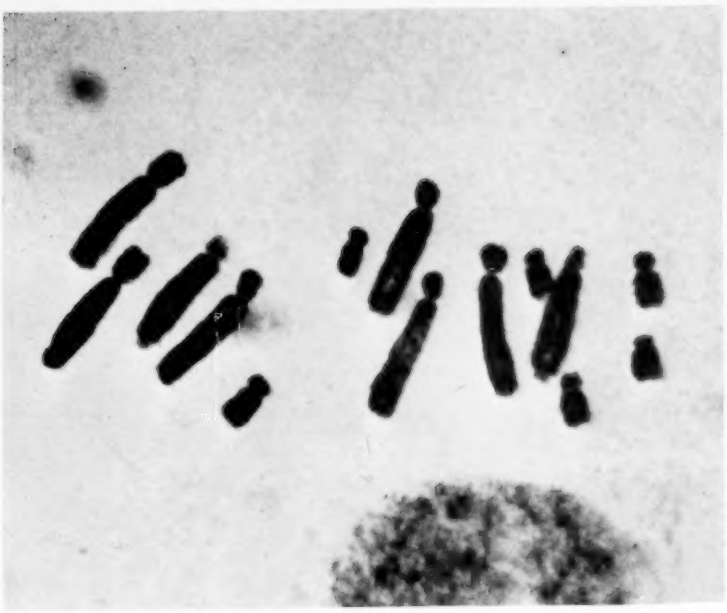

Fig. 1. The 14 or diploid chromosomes in the root-tip of *Aloë Shimperii*; 4 large and 3 small pairs with subterminal constriction indicating the position of the centromere. (Photograph by Mr. B. SNOAD.) ×1900

Fig. 2. The 6 or haploid chromosomes in the pollen grain of *Tradescantia bracteata*. The centromere lies in the middle of the chromosomes. ×2000

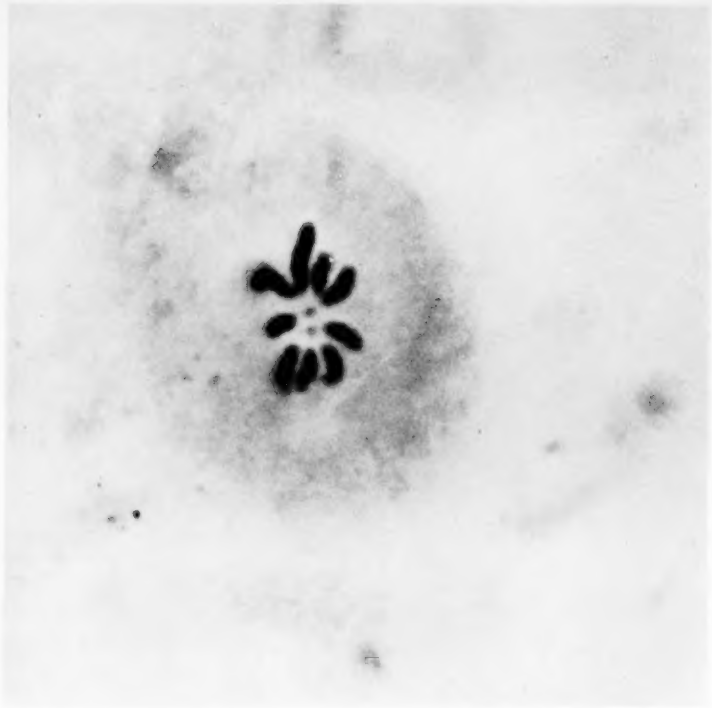

Fig. 3. The 9 chromosomes in the ganglion cell of *Drosophila miranda*; 5 are rod-shaped with a nearly terminal centromere, 2 are dot-like and 2 are v-shaped having a median centromere. The latter represent the sex chromosomes. ×3200

Fig. 4. Two dividing cells in the root-tip of *Vicia* 12 hours after irradiation by 120 r. In the upper cell the 'acentric' fragment is left behind the other chromosomes and lies in the middle of the cell; the lower cell is in telophase and shows the formation of micronucleus. ×1600

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Fig. 5. The 12 chromosomes in the meristem cell of *Vicia faba*: the large *M*-chromosome pair has a median centromere and a nucleolar constriction in one arm; in the 5 pairs of short (S_1 + S_2) chromosomes the position of the centromere is sub-terminal. Each chromosome is composed of two sister chromatids. (Photograph by Dr. S. Revell.) ×2000

Fig. 7. Chromosomes in the pollen grain of *Fritillaria pudica* after 3 weeks of cold treatment showing the understained heterochromatin segments. (Photograph by Mr. L. La Cour.) ×1600

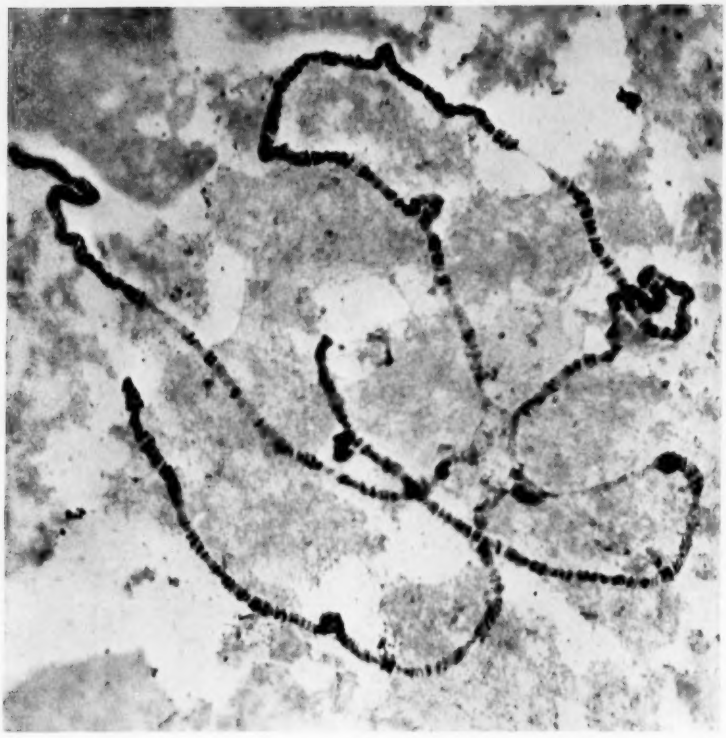

Fig. 8. The polytene chromosomes in the salivary gland of *Drosophila melanogaster*. The heterochromatic sex chromosome and the heterochromatic segments of all chromosomes fuse into the chromocentre. (Photograph by Dr. O. Fahmy.) ×900

Fig. 9. Prophase of mitosis in *Fritillaria falcata* showing the very large Feulgen-positive chromocentre. This species contains almost as much heterochromatin as euchromatin. (Photograph by Mr. L. La Cour.) ×2800

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positive Feulgen reaction (Figs. 9, 10). They are always attached either to the nucleolus or to the nuclear membrane.[4] The variable number

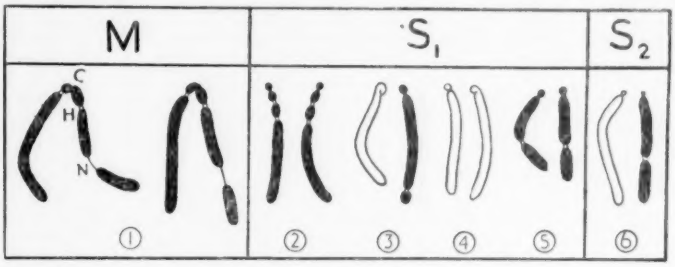

Fig. 6. *Camera Lucida* drawing of the 12 mitotic chromosomes in the root-tip cell of *Vicia*, kept 4 days at 7°C to show the heterochromatin segments. *M*, the long chromosome with median centromere; S_1, and S_2, the short chromosomes with nearly terminal centromere. *C*, centromere, *H*, heterochromatin segments; *N*, nucleolar constriction. Chromosomes not showing heterochromatin are drawn in outline. ×1200. (McLeish[112])

and size of the chromocentres within the same species of cells indicate that they can fuse. In such a case not only the heterochromatin but the adjacent regions of non-homologous chromosomes can come into close contact.

Muller[5] suggested that heterochromatin has originated from euchromatin by a process of progressive degeneration. Recently some evidence had been found to support this view. From the studies of chromosome morphology and behaviour in a tenebroinid beetle (*Tribolium*) Smith[6] came to the conclusion that the heterochromatic sex chromosome originated from one of the euchromatic autosome chromosomes.

The function of heterochromatin blocks in the chromosomes is one of the important problems of cytology and genetics. There is evidence to indicate that heterochromatin regulates the rate of mitosis[7] and the metabolism of nucleic acids[8] and it was also suggested on cytological evidence that alteration in the heterochromatin may be involved in the malignant transformation of cells.[9] The classical studies of McClintock[10] on variegation in maize led her to the conclusion that the heterochromatin plays a role in differentiation and it may represent the controlling system responsible for differential genic action. The distinctive cyclical behaviour of the heterochromatin is attributed to the fact that it is less differentiated internally than the euchromatin.[11]

Crossing over: Controlled Breakage of Chromosomes

The most important phenomenon in the life of the chromosomes is crossing over, i.e. the exchange of parts of chromatids of homologous chromosomes. This process occurs in cells of the germinal tissue during

meiosis, though it may take place in cells of the somatic tissue on rare occasions. The essential feature of crossing over is *the breakage* of two chromatids *at the same locus* which is followed by their fusion in new arrangements (Fig. 11). The exchange of partner chromatids results

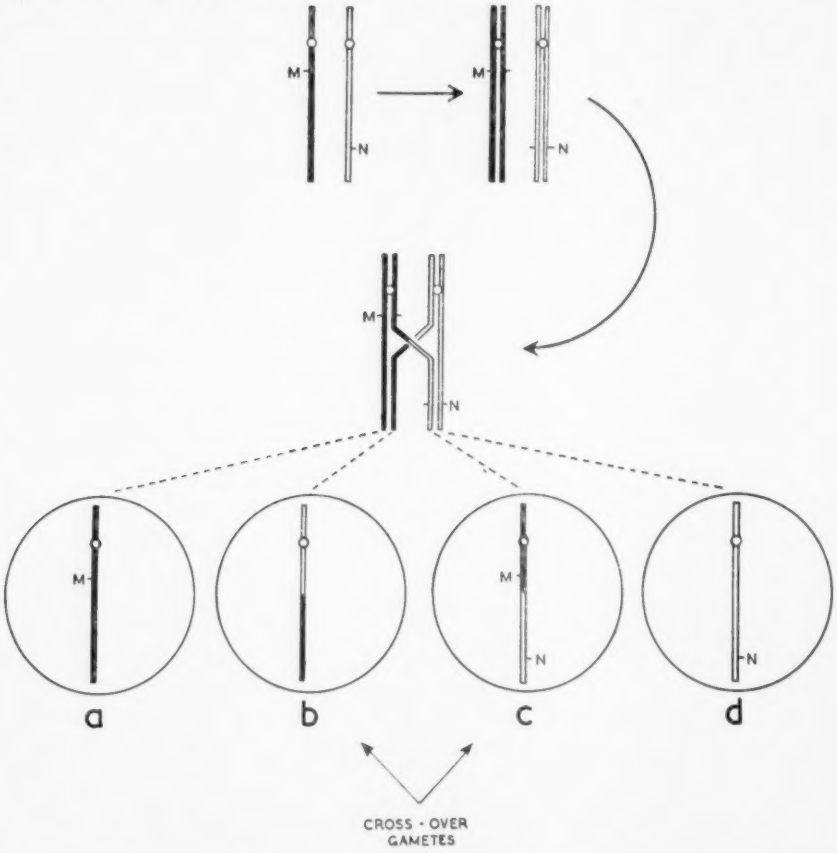

Fig. 11. Diagram showing the sequence of events during crossing over; each of the homologous chromosomes carries a gene *M* and *N* in different positions and as a result of crossing over, gametes (sperms) are produced (*b* and *c*) which have new chromosome types.

in the formation of the *chiasma* which is visible cytologically and is responsible for the metaphase orientation of the meiotic bivalents (Fig. 12). When crossing over occurs at two loci along the chromosome, different chromatids can be involved. The four types of chromatid exchanges are shown in Fig. 13. The loci of breakage are either distributed at random or localized, usually near the centromere.

Crossing over in one region of the chromosome interferes with the

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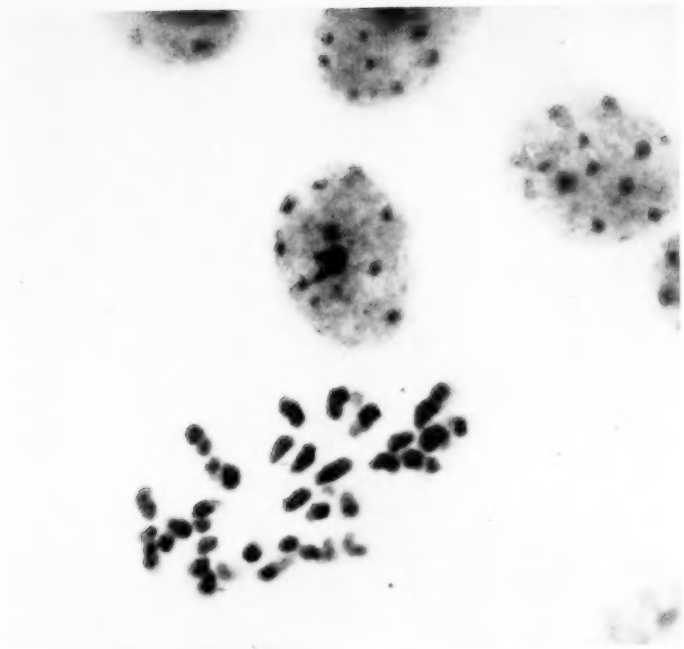

Fig. 10. Metaphase chromosomes of the field-mouse *Apodemus* and three resting cell nuclei showing small but numerous chromocentres. ×1500

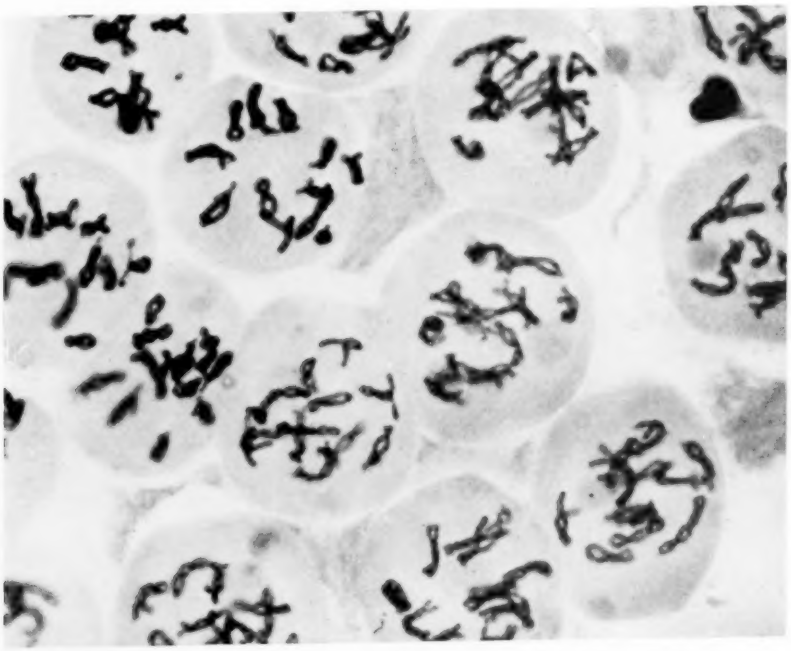

Fig. 12. Pollen mother cells of *Fritillaria pallidiflora* showing the chiasmata between the paired homologous chromosomes. (Photograph by Mr. L. La Cour.) ×1500

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occurrence of crossing over in an adjacent region. There is also interference between chromosomes. The complexity of the inter-chromosomal interference has been shown by SCHULTZ and REDFIELD,[12] who believe that the heterochromatic segments play an important role in determining the extent of interference. Structural differences in the homologous chromosomes act as barriers which either prevent crossing

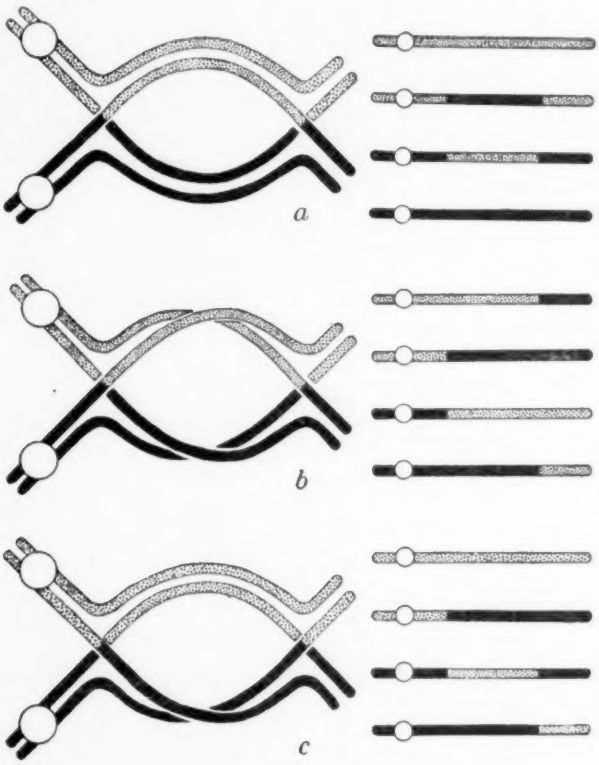

Fig. 13. Diagram illustrating the various types of chiasmata between homologous chromosomes; (*a*) reciprocal, (*b*) complementary and (*c*) diagonal. (From WHITE[14])

over or reduce its frequency. The frequency of crossing over can also be influenced by temperature, ionizing radiation or chemical agents, and it may be under genic control as shown by examples well known in *Drosophila* and plants.

Crossing over coincides with the division of the two paired homologous chromosomes during meiotic prophase. When each of the homologous chromosomes divides into sister chromatids the breakage occurs, which is then followed by fusion. It is possible that the two events are

simultaneous. The phenomenon of crossing over is a natural cyclic breakage of the chromosomes and raises many important questions. Why do only two of the four chromatids break? Why are the two chromatids involved always partners, (i.e. they belong to different members of a homologous chromosome pair)? Why it is that chromatids break at the same level, unequal crossing over being a very rare occurrence? How is genic control over breakage frequency exercised? How can crossing over in one chromosome-pair influence the occurrence of a similar event in other chromosomes?

DARLINGTON[13] made the first attempt to explain the mechanism by which the orderly events of crossing over might be brought about. He attributed them to an excessive force of torsion due to the coiling of the unsplit homologous chromosomes around each other. Under the tension partner chromatids snap, the tension is released by a half-turn and the broken chromatids fuse again.

Another hypothesis as to the cause of crossing over was put forward by WHITE[14] based on the assumption that the splitting of the chromosome thread is not simultaneous along its whole length. He argues that the frontier regions between the heterochromatin blocks and euchromatin—(because of the differences in time of splitting) are favoured loci where breakage of partner chromatids can occur. It seems however, that the cause of crossing over does not lie in the chromosomes alone, the specific physiological environment of the cell in meiotic prophase also plays a role. The importance of the latter led DARLINGTON[15] to formulate the "precocity theory" of meiosis.

Crossing over is a periodic chromosome breakage and reunion, which occurs in every sexually reproducing organism. It is the basis of genic variation and can not only perpetuate, but can also increase diversity on the chromosomal level, e.g. the production and increase in the number of supernumerary chromosomes in the so-called fragmentation and fusion heterozygotes.[16]. Crossing over is an event of the greatest importance in evolution, yet so far no satisfactory analysis has been made of its biophysical and chemical aspects. There is no doubt that a better understanding of the events which result in regular exchanges of broken chromatids during meiosis, would greatly help us in the study of experimentally induced chromosome breakage.

POLYCENTRIC CHROMOSOMES

The movement of chromosomes during mitosis is directed by the centromere, which occupies a permanent position and is endowed with special properties, not shared by other parts of the chromosome. When there is more than one centromere, the orderly behaviour of the chromosome will be upset and the result can be expected to be disastrous for the cell if no special mechanism is adapted to counteract it.

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In one variety of the round-worm *Ascaris* there are two chromosomes in the cells of the germ-line and many (52–72) in the somatic cells. At the first cleavage division the middle region of the two large chromosomes lies on the spindle (Fig. 14). During the following cleavage divisions, the two chromosomes break up into two large segments and many

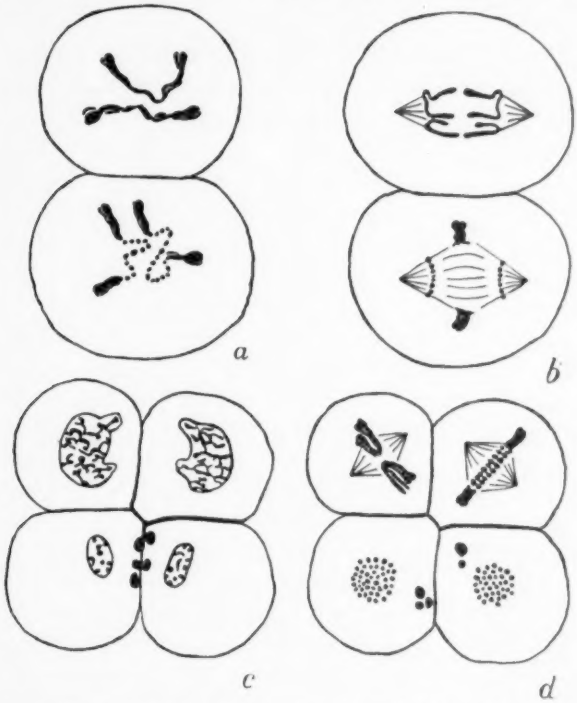

Fig. 14. Cleavage Division in *Ascaris*. (*a*) metaphase and (*b*) anaphase showing the breaking up of the compound centric segment, (*c*) telophase with the eliminated chromatin segments in the two lower cells, (*d*) metaphase of the third cleavage division showing the repetition of the same process in the cell at upper right. (From BOVERY after WHITE[14])

small units. The former behave as acentric fragments and are eliminated while the latter small bodies behave in successive mitosis as independent chromosomes. When the cleavage chromosomes of *Ascaris* are broken by x-rays, only the terminal segments behave as acentric fragments, while the other pieces from the middle part become attached to the spindle at the succeeding division.[17] This behaviour indicates that the two large germ-cell chromosomes are polycentric, having more than one centromere in the middle region. In the cells of the germ-line, these centromeres act as one unit, while in the somatic cell, the specific physiological environment leads to the disruption of the centromere complex.

The "recurrent" fragmentation of one chromosome in the salivary gland of a *Sciara* species is another case of breaking up the compound centromere.[18] There is a "diffuse" centromere in the coccid bug, *Steatococcus* which can be broken by x-rays. The fragments then go through many cycles of somatic division indicating that the components of the compound centromere can act independently.[19]

Dicentric Chromosomes: the Breakage-Fusion-Bridge Cycle

McClintock[20] reported that in a particular maize plant following the breakage of a single chromosome during meiotic prophase, fusion has occurred at the locus of breakage between the two sister chromatids at the time of re-duplication of the chromosome fibre. This results in a chromosome with two centromeres which formed a bridge during anaphase. Because of the tension exerted by the poleward movement of the centromeres, the bridge breaks, thus allowing the two centric products (segments with the centromere) to migrate freely towards the poles. During the following interphase when the broken chromosome undergoes duplication, fusion occurs again between the sister chromatids and the bridge-breakage-fusion cycle continues.

The cycle is restricted to the particular cells of the seed endosperm. In the cells of the embryonic tissue the bridge-breakage-fusion cycle ends, though the "broken" chromosomes are present. McClintock concluded that the broken ends of the chromosomes are healed in these cells, and the healing is permanent.

The breakage of the anaphase bridge is a consequence of mechanical rupture. The type and degree of variegation found in the kernels indicated that the breakage point is not always at the middle of the bridge, though breakage tends to occur at positions of previous fusions. Chromosomes with duplications and deficiencies are produced with certain frequencies as a result of the "un-equal" or non-median breaks. The survival of cells with abnormal chromosomes is due to the peculiar genetic and physiological conditions of the triploid endosperm tissue.

In a variety of Daffodil (the triploid *Narcissus pseudo-narcissus*) a dicentric chromosome persisted for four years by the bridge-breakage-fusion cycle.[21] Though during that time the dicentric chromosome must have broken thousands of times, the variation in the intercentric segment (part lying between the two centromeres) was slight, which indicates that the break in the anaphase bridge is mostly median so that the two centric products are alike in length. Any loss which may have occurred by unequal break in the bridge is compensated by the presence in the same cell of two other homologous chromosomes.[151]

Another instance of the bridge-breakage-fusion cycle has been found in a rat sarcoma (Fig. 15–17). The tumour was the result of repeated

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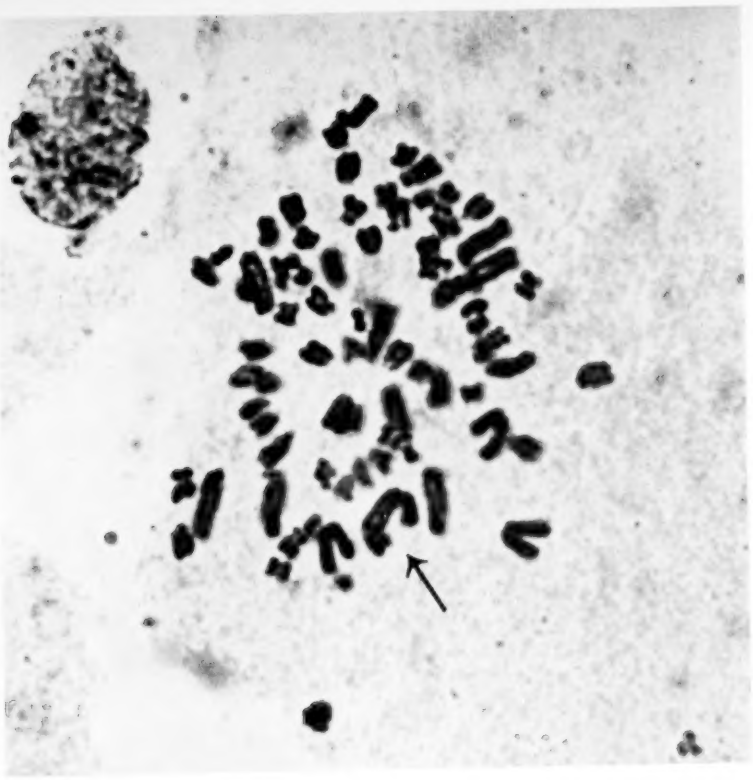

Fig. 15. Metaphase chromosomes of a nitrogen mustard induced sarcoma in the rat. The large chromosome at 6 o'clock has two constrictions and represents the dicentric chromosome. ×1800

Fig. 16. Double chromosome bridge formed by dicentric chromosomes. (KOLLER[23].) ×2000

Fig. 17. Telophase of mitosis showing the mechanical breakage of the dicentric bridge. The broken ends come into close contact and fuse again. (KOLLER[23],) × 2000

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Fig. 20. Late anaphase of mitosis in a rat sarcoma showing three sticky bridges. × 2000

subcutaneous injections of an aromatic nitrogen mustard.[22] The behaviour of dicentric chromosomes has been followed through more than a hundred transplantations and was analysed in detail by the author.[23] In the primary tumour, which developed at the site of injections, about 68·5 per cent of the dividing cells had anaphase

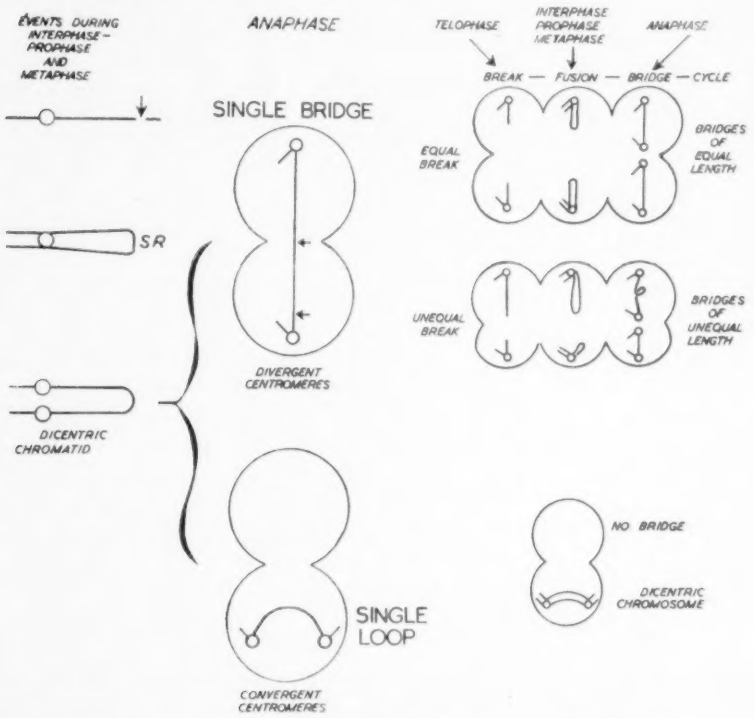

Fig. 18. Diagram illustrating the origin of dicentric anaphase bridges and the sequence of events in the break-fusion-bridge cycle. (KOLLER[23])

chromosome bridges. The number, shape and length of the dicentric bridges showed a very wide range of variation. Some cells were free of bridges, some had only one while in others the number of bridges increased to ten or even more.

The different lengths of bridges are attributed to the variable position of the breaks which occur during telophase (Fig. 18). Breaks in the middle of the intercentric segment were just as common as breaks near the centromere. In a few cells, which had no bridges, a loop-chromosome was found, which represents the dicentric chromosome, whose two centromeres migrate towards the same pole. During interphase the unbroken loop chromosome divides or "splits". At anaphase

of the next division a double bridge will be formed, which can be of two types; one is the criss-cross, the other is the interlocked bridge. The origin of the various anaphase configurations of the dicentric chromosome bridge is illustrated in Fig. 19.

When the intercentric segment breaks at anaphase, the two monocentric products, whose centromeres are oriented towards the same pole,

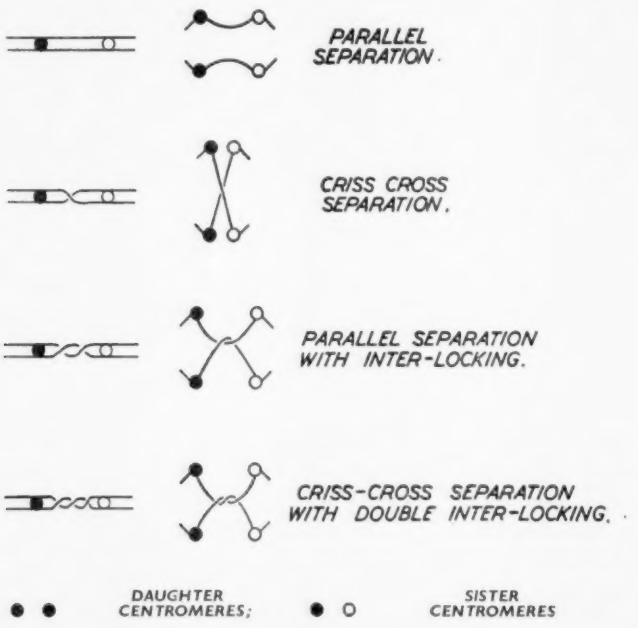

Fig. 19. Diagram showing the various configurations of dicentric chromosomes at anaphase. (KOLLER[23])

move together into the telophase chromosome group. By doing so their broken ends come into close contact, which naturally favours their fusion. Thus at the end of telophase another dicentric chromosome is formed which repeats again the bridge-breakage-fusion cycle.

Dicentric chromosomes are observed in many plants and animals; some of the cytologically studied cases are listed in Table 1.

As far as anaphase behaviour is concerned, *ring chromosomes* (chromosomes with no ends) may be classed together with the dicentrics. They are found in *Drosophila*[24] and have been studied in detail in maize.[25] When the ring chromosome splits, either two rings of the same size are formed, each with one centromere, or frequently a double-sized ring is the result, which has two centromeres. At the next anaphase, owing to the centromeres moving to opposite poles, the double-sized ring is

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TABLE I

The occurrence of dicentric chromosomes[21]

	Induced	*Spontaneous*
Temporary	*Crocus* (MATHER and S. STONE, 1933) *Pisum* (KOLLER 1934) *Allium*, *Tradescantia* (SAX 1941) *Drosophila* (PONTECORVO and MULLER, 1941)	Pollen grain divisions in many plants; e.g. *Tulipa* (UPCOTT 1937) Primary divisions in seedling roots; e.g. *Crepis* (NAVASHIN 1933) *Allium* (NICHOLS 1941) *Pisum* (D'AMATO 1951)
Persistent	? *Tulipa* (2x) (MATHER and STONE 1933) *Maize* (2x) (MCCLINTOCK 1942–3) *Axolotl* (2x) (DALTON and HALL 1950)	*Godetia* (HAKANSSON 1930)
Permanent	*Rat* tumour (KOLLER 1952)	*Scilla* (BATTAGLIA 1949) *Agropyron* (HAIR 1952)

stretched and breaks at two places. Fusion of the sister chromatids during interphase initiates a new cycle.

The behaviour of dicentric bridges shows that they break *under tension*, exerted from the poleward migration of the centromeres. The breakage represents a *mechanical* disruption of the chromosome fibre, which is followed by fusion of the broken ends. The two events are separated in time.

False Dicentric Bridges

Anaphase bridges are common abnormalities in tumour cells and in cells which have been x-rayed and fixed a few hours after irradiation for cytological analysis.[26] These bridges are formed by the daughter chromosomes which stick together at their ends (Fig. 20). They may part again at telophase when the chromosomes are stretched between the two poles. But instances are frequent where the bridge persists throughout telophase and resting stage. If more than one chromosome pair is involved, the "bridge" will be a solid, thick column, which does not break apart and which is responsible for the characteristic dumb-bell shaped compound nucleus. The breakage in the sticky bridge is a mechanical disruption of the chromosome and it usually occurs at the locus where the chromosomes have stuck together. Frequently the intercalary segment, which lies between the centromere and the point of stickiness, is stretched by the force of tension without the chromosome being broken. Then at the end of telophase, the thin and attenuated

chromosome fibre is attacked by the nucleolytic or proteolytic enzymes of the cytoplasm, and as a result the bridge disintegrates (Fig. 21).[27]

The sticky bridges are difficult to distinguish from the true dicentric bridges described previously. Discrimination is possible only if the chromosomes stick together at some distance from the end and especially if the stickiness is restricted to a small intercalary section. The configuration which results from this kind of stickiness may be described as "pseudo-chiasma" (Fig. 22).[23], [28] The chromosome deficiency, which results when an intercalary sticky bridge breaks, has fatal consequences and the cell dies. There is no evidence, except for a few instances observed in malignant cells, that the broken chromosomes, which formed a "sticky bridge," could initiate a bridge-breakage-fusion cycle.

It has been suggested by the late Professor J. M. GULLAND that stickiness of the chromosomes may be brought about by the cross-polymerization of the deoxyribose nucleic acid. DARLINGTON[29] attributes stickiness to an excess of nucleic acid charge on the chromosomes. Chromosome stickiness occurs in nature and it can be produced experimentally by changes in temperature,[30] by chemical agents[31], [32] and by supersonics.[33] This phenomenon is particularly common in tumour cells,[27] and may be attributable to some change taking place in the cytoplasmic environment when a cell becomes malignant. Stickiness of chromosomes can also be a genetically controlled abnormality. Such a case was found in maize.[34]

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MISDIVISION OF THE CENTROMERE

The division of the centromere at the end of metaphase occurs *longitudinally*, i.e. in the plane which is perpendicular to the spindle or polar axis (Fig. 23). The separation of daughter centromeres and chromosomes takes place in this plane. When the plane of the centromere division is *transverse* then instead of the normal anaphase separation, both the centromere and chromosome break up. This event is described as misdivision of the centromere. As a result new telocentric chromosomes appear, which represent the different arms of the original chromosome. Misdivision of the centromere is known to occur only in the univalent or unpaired chromosomes during meiosis. This phenomenon was discovered in *Tulipa*.[35] A similar abnormality was observed in *Pisum*[36] and the consequences were studied in *Fritillaria*[37] and in common wheat.[38]

By using 8-oxyquinoline as a fixing medium, LEVAN and TJIO[39] succeeded in demonstrating the structure of the primary or centric constriction in the chromosomes of various plants. They found 4 centromeric chromomeres which appear as small Feulgen positive granules. It seems that during "misdivision" the 4 component elements

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Fig. 21. Telophase of mitosis in a human skin carcinoma with a long, thin sticky bridge. ×2400

Fig. 22. Anaphase in *Vicia*, one hour after 100 r showing the "pseudo-chiasmata" due to chromosome stickiness. (Photograph by Dr. S. REVELL.) ×1600

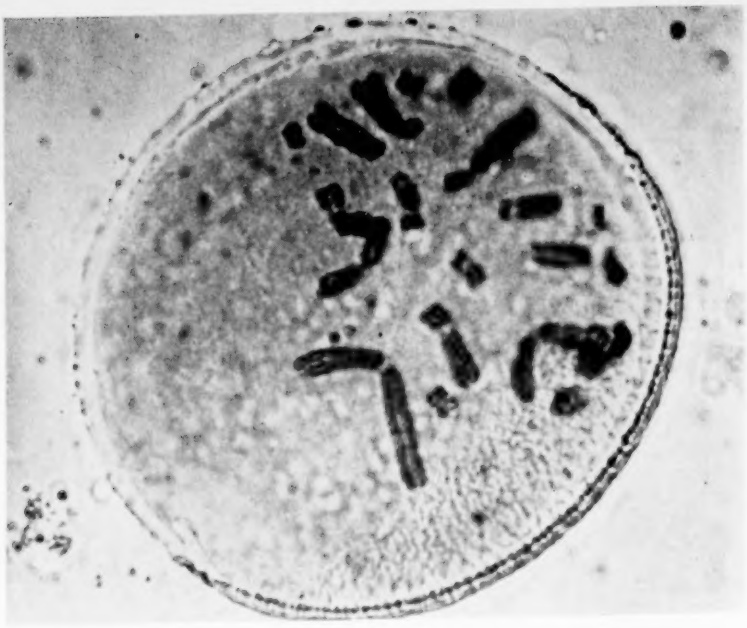

Fig. 24. Pollen-grain of *Tulipa fragrans*, in which 10 out of the 12 chromosomes have been broken; there are several acentric fragments and one dicentric chromosome. (DARLINGTON and UPCOTT[401].) ×1200

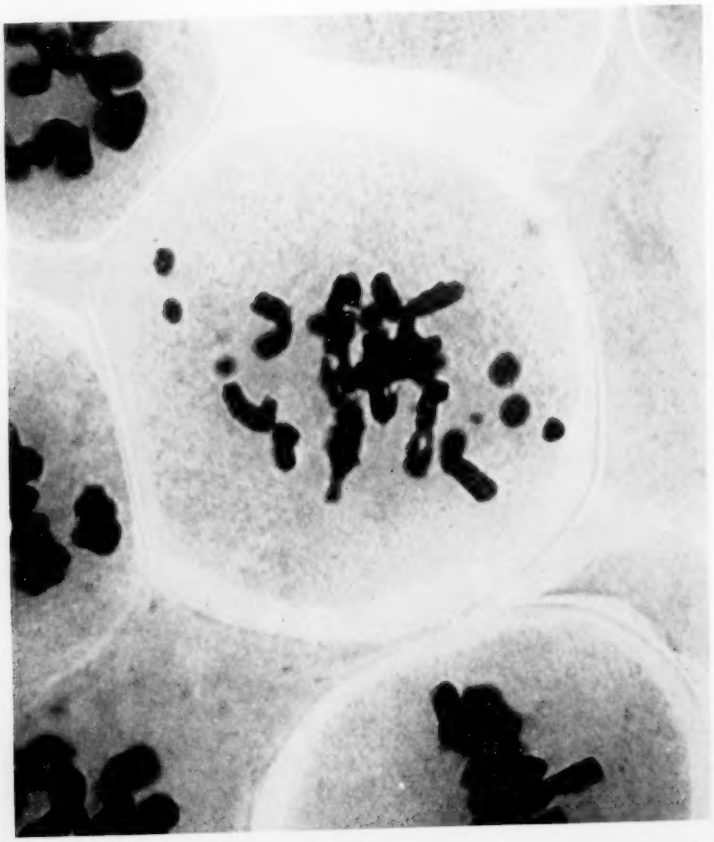

Fig. 25. Meiosis in *Allium cepa* showing spontaneous breakage. (Photograph by Mr. A. HAQUE.) ×1500

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of the centromere-complex become separated. The abnormal division of the centromere must be considered as a special event and it is very likely due to mechanical as well as physiological conditions which operate in a univalent chromosome during meiosis.

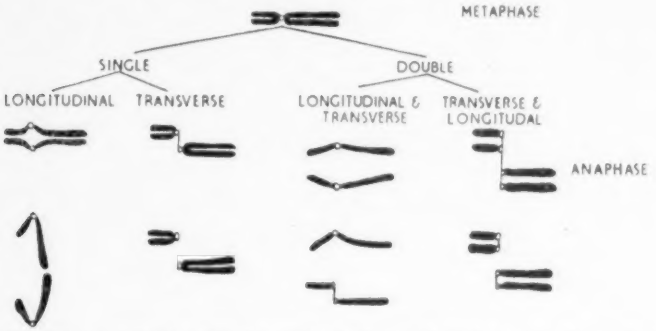

Fig. 23. Diagram illustrating the method of normal and abnormal centromere division. (KOLLER[36])

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SPONTANEOUS OR NATURAL BREAKAGE OF CHROMOSOMES

In the previous part I described special cases of chromosome breakage, in which it was restricted to particular chromosomes or chromosome parts with a predictable outcome. However, there is another class of chromosome breakage which occurs under natural conditions, the cause of which is obscure, and the consequence of this event cannot be predicted.

Natural breakage of chromosomes has been observed in many plants and animals, in different cells and tissues (Figs. 24, 25 and 26). Fragmented chromosomes were observed, e.g. in the cells of the meristem tissue, in roots, in cells undergoing meiosis, in pollen grains and pollen tubes, in tumours, etc.

Chromosome fragmentation on a rather excessive scale was found in *Tulipa*, *Hyacinthus* and *Tradescantia*. The various chromosome configurations, which resulted from breakage, have been analysed in detail by DARLINGTON and UPCOTT.[40] Their interpretation was based on two important principles: (*i*) that breakage occurs first, followed by restitution or reunion and (*ii*) that the chromosomes can be broken before or after they divide. The events of individual cells were analysed and variability between cells compared. They introduced a new terminology and symbols to indicate the various events responsible for a particular chromosome configuration. Bodies of chromosomes were labelled according to the number of centromeres present: C_0 = acentric, C_1 = monocentric, C_2 = dicentric, etc. Distinction was made between chromatid and chromosome breaks: B' and B''. Reunion of sister

chromatids is denoted as SR, and distinguished from chromatid or chromosome reunion: R' and R'' (Fig. 27).

Many important features of chromosome breakage were revealed by the new method of analysis which takes into consideration the intra- and inter-cell differences. The empirical combination of broken chromosomes from which breakage and reunion are inferred, was found to show no linear relationship with either component. It was also detected that SR (reunion of sister chromatids) is disproportionately

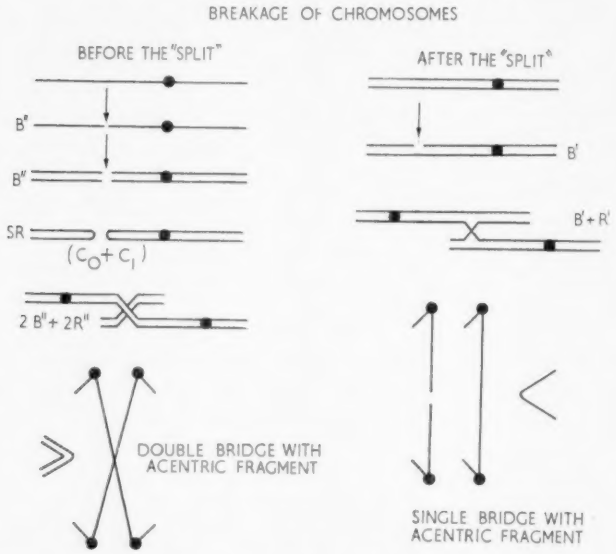

Fig. 27. Diagram illustrating some of the configurations which can arise after the breakage of a chromosome or chromatid.

frequent in cells with many broken chromosomes. The very small acentric fragments or "minutes" were shown to be due to two breaks which occurred in close proximity and followed by reunion of the two broken ends thus preventing SR. They demonstrated furthermore that competition between *restitution* (rejoining of broken chromosomes in the original) and *reunion* (rejoining of broken chromosomes in a new way) which comes into operation after breakage, is an important element of structural change.

The natural breaks of chromosomes in the meristem cells of *Vicia*, are always symmetrical, i.e. both sister chromatids are broken at the same level, indicating that the event took place before the "splitting" or duplication of the chromosome during the resting stage. It was also found that breaks are crowded near the centromere, and the short chromosomes are affected more than the long M chromosome.[41]

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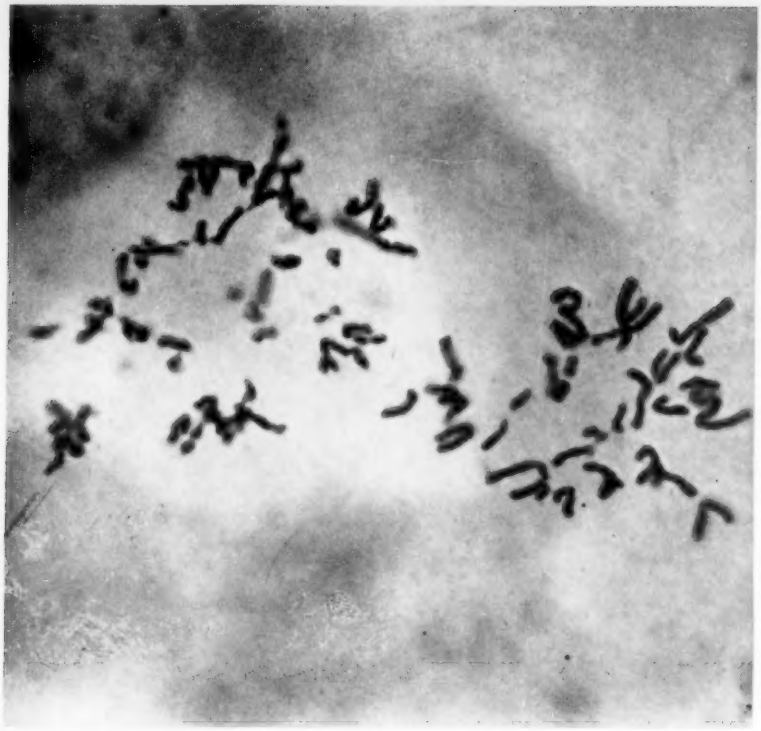

Fig. 26. Two dividing cells in an adenocarcinoma of man; the chromosomes of the cell on the left are undergoing spontaneous breakage. ×2000

Fig. 28. Meristem cell of *Vicia* 12 hours after 130 r., showing chromatid break (B') and chromatid interchanges ($2B' + 2R'$). ×1600

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Many other examples could be mentioned to show that natural breakage is rather a common occurrence, but usually the damage is so drastic that the affected cell rarely survives. If, however, the breakage does not involve loss, reunion may occur forming new chromosomes which can then function. Natural breakage no doubt played and still plays an important role in evolution.[42]

The question arises: what is the cause of the "spontaneous" breaks? GILES[43] has shown that in *Tradescantia* they could not be brought about by natural or cosmic radiation. The natural radiation level is about a thousand times too small to account for the spontaneous chromosome breakage rate. Intracellular metabolic disturbance, due to enviromental factors (e.g. changes in oxygen tension, in temperature, etc.) are most likely the immediate cause of such events in cells. How such metabolic disturbance or cytoplasmic instability may be brought into operation is illustrated in the case of *Kinugasa*, a Japanese lily.[44] It grows in subalpine regions of the mountains in the north of Honshu, the main island of Japan. When the plant is transplanted from its native place to the temperate south it shows drastic chromosome disturbances, fragmentation being most common. These irregularities are attributed to the environmental change in temperature and nutrition, which are bound to affect the intracellular conditions. The stability of the latter is precariously balanced, particularly in species hybrids, triploids and tumours in which natural breakage of chromosomes is frequently observed.[43], [45], [46], [47] It seems that some error in cell metabolism, whatever the primary cause may be, is a major factor in the chromosome breaks.[48] REES[49] found in *Scilla* that cells with broken chromosomes are much larger than normal cells, suggesting that protein synthesis has been favoured at the expense of nucleic acid (DNA) synthesis. Examples are known in which the fragmentation of chromosomes and their susceptibility to breakage are controlled by genes. Such genes probably act by altering cell metabolism.[50]

Spontaneous breakage of chromosomes was also seen in differentiated cells. Mitosis in such cells is extremely rare, but by application of various growth-substances, e.g. 2·4-dichlorophenoxy acetic acid, indol-3-acetic acid and naphthalene-acetic acid, division of differentiated cells of the pericycle tissue in roots can be induced, and it has been found that they contained many broken chromosomes. A similar phenomenon has been observed in ageing tissues.[51], [52]

The breaking up of the chromosomes in differentiated cells was attributed to "physiological decay," i.e. to the greatly altered metabolic conditions within the cell. D'AMATO[53] provided evidence that the metabolites of differentiated cells under certain conditions may act as promoting agents, and induce chromosome fragmentation in cells of the adjacent meristematic tissues.

The natural breakage of chromosomes is brought about by the response of cellular metabolism to environmental influences. From the evolutionary aspect it is an event of great importance, and in the study of the experimentally induced chromosome reaction it is of fundamental significance.

Radiation Induced Breakage

The discovery that irradiation produces mutations, i.e. permanent changes in the genes[54] was followed by the discovery that it can produce breaks in the chromosomes.[55] The latter event was inferred from genetical analysis and from the new configurations which have been formed by the broken chromosomes. Geneticists found a definite quantitative relationship between single ionizations and gene mutations. With the discovery of the salivary gland chromosomes in *Drosophila* more valuable information were obtained. It was seen that many of the genetical changes were due to structural changes in the chromosomes such as deletions, inversions, interchanges, etc. These changes represent new rearrangements brought about by the reunion of the broken chromosome ends. Attempts have been made to relate the complex end-results to direct dosage effects. The limitation of such a quantitative analysis is obvious for various reasons.

The number of the initial breaks, which reunite in the old way, i.e. *restitute*, cannot be ascertained; they are lost to observation. Many initial breaks are eliminated because they reunite and form such complex configurations that they cannot survive beyond the first mitosis. The observed breaks in the salivary gland chromosomes represent only a small fraction of the initial breaks, i.e. those which formed viable rearrangements, and were able to survive the thousand mitoses of larval development.

1. *Quantitative analysis of chromosome breakage*

The introduction of suitable cytological material (pollen grains of *Tradescantia*, meristem cells of *Vicia* (Fig. 28)) and of improved techniques led to the study of the chromosome injuries, at the *first* mitosis after irradiation.[56–60] Meanwhile the "target-hypothesis" has been elaborated and has become the only basis of interpretation of genetical experiments. The tendency was therefore natural to extend the validity of this hypothesis to chromosome rearrangements. Though they are subsequent to breakage, they were treated as comparable to the initial effects. The three phenomena, (*i*) ionization, (*ii*) breakage of the chromosome and (*iii*) the reunion of broken chromosome ends, are causally related but are distinctly separated events in time.[61] The x-rayed chromosomes of the *Drosophila* sperm present a good example. Breakage occurs at the time of the "split" or duplication of the

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chromosome thread. It may be caused by the faulty reproduction of a gene which has been injured by the radiation hours, days or perhaps weeks before. Reunion of the broken chromosome ends occurs after the sperm nucleus has entered into the cytoplasm of the egg.

Efforts have been made by physicists and cytologists[62–69] to find simple numerical formulae by which the various consequences of single ionizations could be explained and the frequencies of the new chromosome configurations could be predicted. The chromosome breaks are treated and analysed as "mechanical" effects, the direct results of single events of ionization. The fact that the chromosomes are not only physical entities, but dynamic components of a physiological system which is in a continuous flux during the developmental cycle, has been conveniently omitted from the calculations. The possibility that irradiation can alter the time-scale of cell development was not considered. Generalizations were made on observations obtained from chromosome behaviour in sperms, pollen grains and pollen tubes, the haploid condition of which limits the analysis from the cytological aspect. The proportionality of lethal gene mutation-frequency to dosage was shown to be due to a "composite effect" produced by the combination of one- and two-hit events. Because there are no criteria by which the expectation of the various types of rearrangements can be calculated, it is not possible to determine how much each event contributes to the composite effect. The pioneer work of STADLER[70] on ultraviolet-induced genetical effects demonstrated that the breakage induced by x-rays and ultraviolet is qualitatively different. Yet, in spite of these observations, the conclusion has been drawn that the relation between irradiation and chromosome breakage is simple and fundamentally similar to that between irradiation and gene mutation.[71] On this assumption a number of mathematical equations, which applied to gene mutations, were adapted to explain chromosome breakage. The calculations were "given in such a mathematically technical and elided form that most biologists reading them—and they must have been intended for biologists—had to take the conclusion 'on faith'."[72] Attention has been focussed on the breakage, and the qualitative aspect of breakage and reunion of broken chromosomes was treated as an event of secondary importance.

A quite different attempt to interpret the cytological phenomena following irradiation was made by DARLINGTON and LA COUR.[73] Their analysis was based on the spontaneous chromosome changes. The radiation effects were studied in samples of complete cells in complete developmental series using meristem cells of root-tips, pollen grains and pollen tubes with resting stages lasting from a few hours up to 195 days. The breakage and reunion of chromosomes were recorded separately as two distinct events. Doses as low as 5 r up to 360 r were

used over a wide range of controlled temperatures. It was shown that when an average of total cell samples is considered, breakage of the chromosomes varies directly with dose; on the other hand, taking not whole cell samples, but cells in classes with different numbers of breaks, reunion of broken chromosome ends varies with breakage. DARLINGTON and LA COUR's analysis has demonstrated the complex nature of the radiation reaction within the cell, which has been further emphasized by the fact that the damage caused by radiation can be reversed by infrared radiation long after the cell has been irradiated.[74],[75] It has also been shown that the altered molecule can diffuse and produce its effects in another part of the cell, thus causing perhaps a thousand-fold increase in the effectiveness of a single ionization.[76] Thus, the once so hotly debated point as to whether the ionizing particle does actually traverse the chromosome thread or pass "near" to it, loses importance. Recent observations have already necessitated many modifications of the earlier concept of chromosome breakage and in view of the above facts, the search after simple numerical formulae becomes indeed a hopeless task.[151]

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2 *Combined treatments and chromosome reaction*

The many important developments in radio-biology have been surveyed by GRAY[77] in a previous volume of *Progress in Biophysics*. He collected a large volume of evidence which shows that for the development of a radiation injury, be it cytoplasmic or nuclear, the metabolism of the whole cell is involved.

X-rays produce a wide variety of changes in the cell and chromosomes. The potentialities of these changes vary, some may undergo repair, others produce lesions and injuries (breaks in the chromosomes), while others may require activation (infrared or temperature shock) to become effective. It has been claimed that infrared and x-rays induce metastable states in the chromosomes which can be reversed by temperature shocks. Such labile states are known to occur in crystals after irradiation. By using infrared radiation, SWANSON and YOST[78] "energized" the state of the *Tradescantia* chromosomes, which then became more fragile to subsequent irradiation with x-rays. The frequency of the initial breaks was significantly raised by such double treatment. On the other hand when x-rays and ultraviolet (2537Å) are combined, it is not the initial breakage frequency but the frequency of reunion of the broken chromosomes which is increased.[79], [80]

There is evidence that irradiation may affect the initial breakage frequency as well as the reunion of broken ends. LANE[81] fractionated the dose and varied the time interval between two fractions and found that breaks in the chromosomes of *Tradescantia* pollen grains decreased, as the period between fractions is increased up to 4 hours (Fig. 29).

Beyond that the frequency increased again and at 8 hours separation is little below that from a continuous dose. LANE concluded that radiation affects the sensitivity of the chromosomes and that reunion or

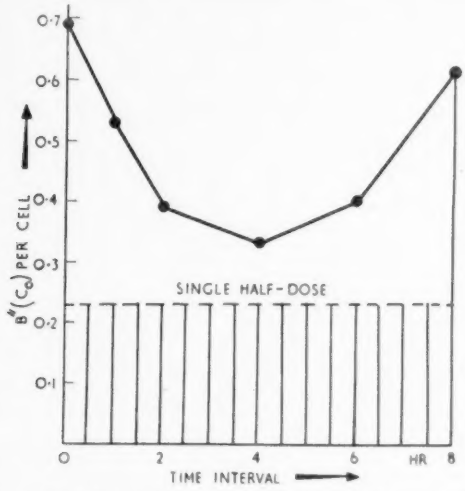

Fig. 29. Frequency of chromosome breaks B'' (or C_0) 72 hours after 360 r x-ray dose in relation to the time interval between two equal fractions. (2 × 180 r) (From LANE[81])

restitution of chromosomes does not follow breakage immediately; these events are separated in time.

3 *Chromosome behaviour during low intensity radiation*

When the chromosomes of *Tradescantia* are exposed to very low intensity radiation (200 r at 0·1 r/min.), mitosis is not suppressed during the treatment[82], [83] (Fig. 30) and it is possible to analyse chromosome behaviour at the time when the "primary" or physiological reactions usually appear in the cell. Both spurious and true chromatid breaks may be present in the same cell, though they were initiated at a different developmental stage (Fig. 31). In the production of chromatid and chromosome breaks ($B' + B''$) the duration of irradiation seems to be more important than the dose.

It was found that 400 minutes continuous radiation (at 0·1 r/min.) is required to produce B' and 600 min., to yield B''. This fact shows that breakage in the chromosome filament is closely linked with a definite stage of development. With low intensity radiation the frequency of mitosis in the anthers of *Tradescantia* has significantly increased (Fig. 32). On the other hand the relative frequency of the different mitotic stages remained the same, indicating that the increase is not due to arrest of metaphase or anaphase, but due to more cells entering

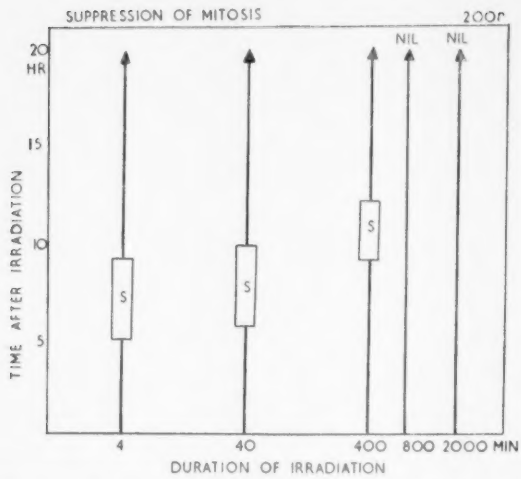

Fig. 30. Diagram illustrating the effect of 200 r at different intensities on mitosis in the pollen grains of *Tradescantia*. The period of mitotic suppression (*S*) is shown. (KOLLER[83])

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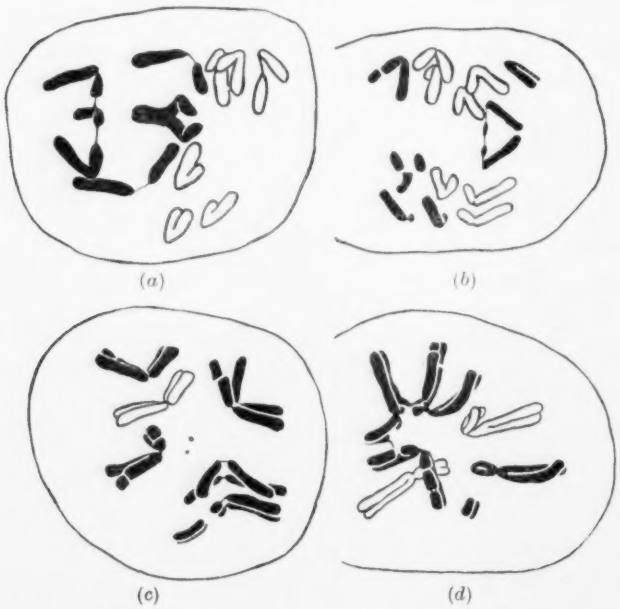

Fig. 31. Camera lucida drawings of *Tradescantia* pollen grains at the end of low intensity radiation; (*a*) centromere effects and stickiness (200 r/400 min. duration), (*b*) spurious and true chromatid breaks (200 r/800 min.), (*c*) chromosome and chromatid breaks with minute fragments (100 r/1000 min.), (*d*) chromosome breaks with SR and incomplete chromatid interchanges (200 r/2000 min.). (KOLLER[83])

TABLE 2

Times at which different cytological effects are observed after irradiation with x- and γ-rays at low dosage rate. (Anthers are fixed at the end of treatment: 0 hour)[83]

Duration of irradiation (minutes)	*Dose*	*Stickiness*		*Delay of metaphase*	*Errors of spindle*	*Errors of spiralization*		*B′*	*B″*	*Reunion*	*Micronuclei*
		Met.	*Ana.*			*Met.*	*Ana.*				
100	50 r	+++	++†	..	..	..	..	..	..	..	..
200	50 r	++†	+*	..	..	..	..	..	..	..	..
200	100 r	+++	++	+	..	..	..	..	..	..	..
300	150 r	+*	++	..	..	..	..	..	..	..	..
400	100 r	+	+	..	..	..	..	..	..	..	..
400	200 r	+	++	+	+	..	+	+	..	R′	..
500	50 r	..	+	+	..	..	+	+	..	..	..
600	150 r	+*	+	+	+	+	+	+	+	..	..
800	200 r	+	+	+	+	..	+	+	+	SR, R′, R″	+
1000	100 r	..	+	+	..	+	+	+	+	SR, R′	+
1500	150 r	..	+	..	..	+	+	+	+	SR, R′	+
2000	200 r	+	..	..	..	..	+	+	+	SR, R′, R″	+

Note.—Frequency of cells showing stickiness is indicated as follows:
+ : less than 10 per cent of P.G.
++ : between 10–20 per cent.
+++ : more than 50 per cent.
X-rays gave more stickiness than γ. † X-rays gave less stickiness than γ.

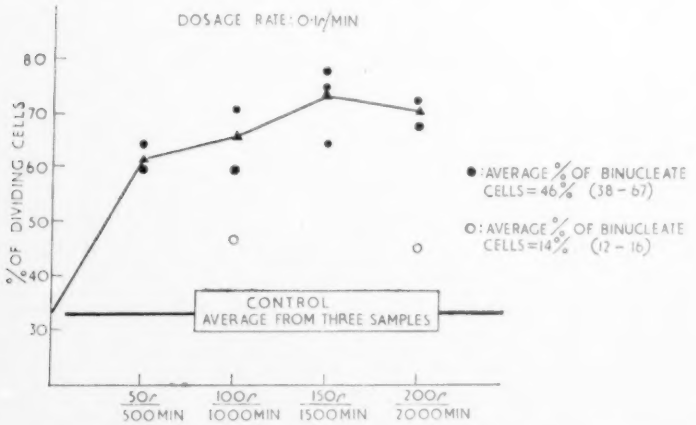

Fig. 32. Graph showing the frequency of dividing pollen grains in *Tradescantia* at the termination of irradiation with 50, 100, 150 and 200 r. Young anthers (with 14 per cent binucleate pollen grains) are less affected than older anthers (with 46 per cent binucleate pollen grains). (KOLLER[83])

prophase. Cell development proceeds but there is usually a change in the sequence of events following low intensity radiation. The physiological effects are superimposed on the genetical effects and both appear together. Any distinction therefore between "primary" and "secondary" effects of radiation becomes meaningless. The fact that at low intensity radiation the "physiological" effects are delayed in the cell, while the rate of mitosis is increased, suggest that protein synthesis and nucleic acid metabolism may be differentially affected. The altered rate of uptake of P^{32} and S^{35} after irradiation shows that his may be the case.[84]

4 *Differential sensitivity*

The differences in the radiosensitivity of various organisms, tissues or cells is a firmly established fact.[85] The most important difference in

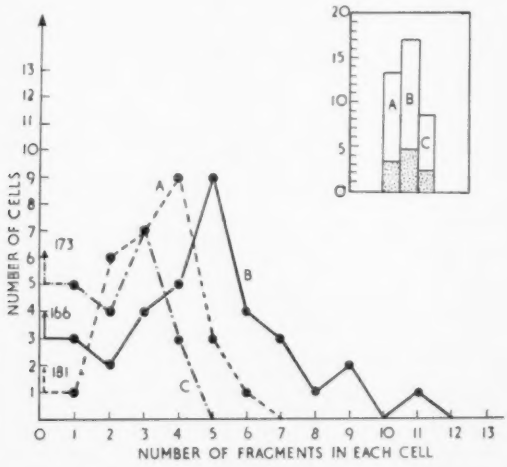

Fig. 33. Graph showing the frequency distribution of cells with chromosome injuries in three carcinomas of the skin (*A*, *B*, *C*) 22 hours after 300 r. Inset shows percentage of abnormal cells and the mean number of chromosome fragments per cell.

sensitivity exists between the cells. This is shown by the fact that the same radiation dose may produce significantly different breakage frequencies in the same kinds of cells. It was found for instance that the distribution of fragments per cell and the percentage of cells with chromosome abnormalities differed in three skin carcinomata, analysed 22 hours after irradiation with the same dose (Fig. 33). By using the breakability of chromosomes as a measure of sensitivity, it has been observed that as the cell proceeds towards the next mitosis, breakability increases and rises to a peak immediately at the end of resting stage or beginning of prophase[73], [86] (Fig. 34). The time of high sensitivity

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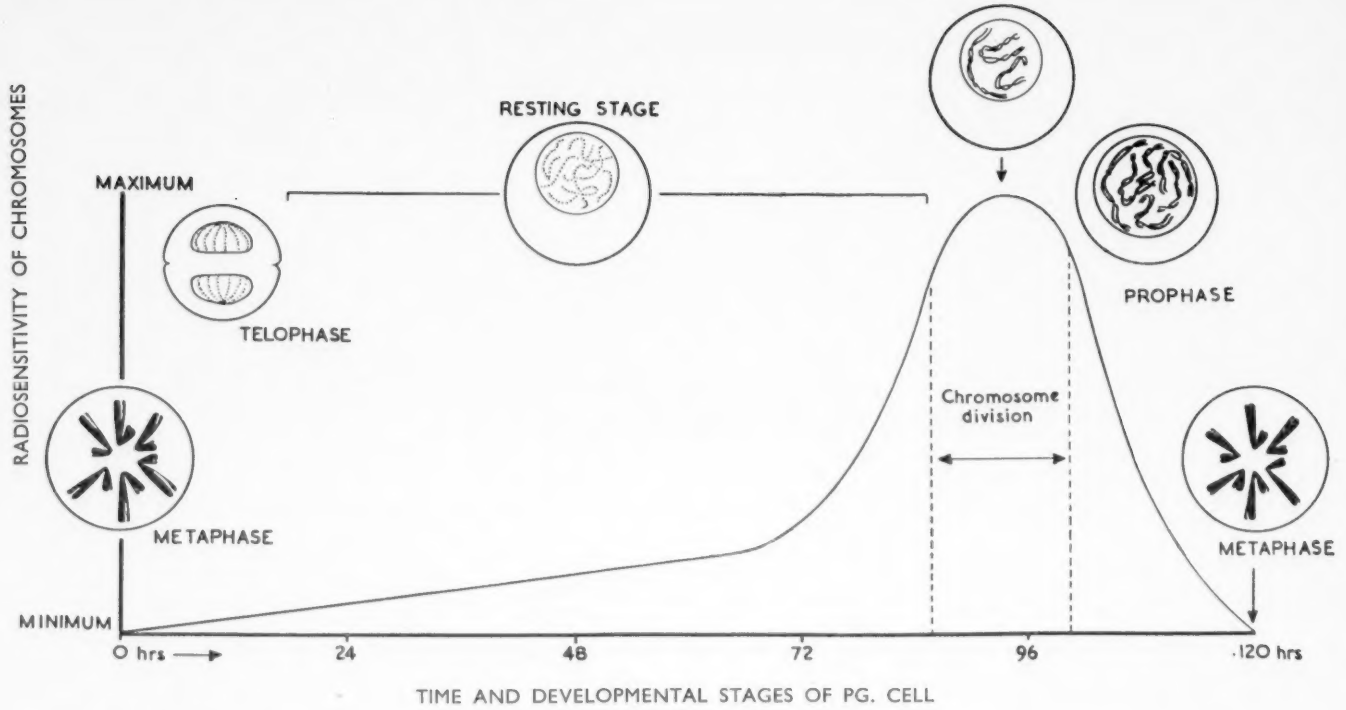

Fig. 34. Diagram showing the mitotic stages and the sensitivity of chromosomes in *Tradescantia* pollen grains (PG). (KOLLER[82])

apparently coincides with the "splitting" or duplication of the chromosomes.

There are also reports of regional differences in the sensitivity of chromosomes to x-rays. Particular loci (e.g. centromere) or segments of chromosomes in certain instances can show a much higher breakability than other parts. Thus KAUFMANN[87] counted more breaks in some regions of the *X* chromosome in *Drosophila* after x-rays then were expected on random distribution. All these regions contained heterochromatin blocks. SAX[65] drew attention to the fact that x-ray breaks

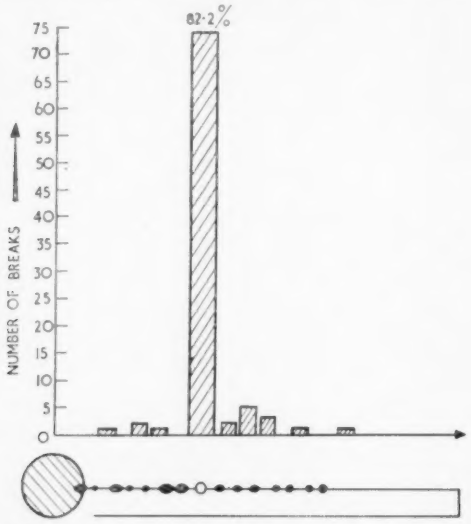

Fig. 35. Distribution of x-ray induced breaks in 90 nucleolar chromosomes of *Solanum*. 82·2 per cent of the breaks are located in the centromere region. (From GOTTSCHALK[88])

in *Tradescantia* are very frequent in the region near the centromere. Similarly, in the plant *Solanum*, GOTTSCHALK[88] found the centromere region to be especially sensitive of x-ray breakage (Fig. 35). He also observed a higher breakage frequency in the heterochromatin segments than in the euchromatin regions. The differences in sensitivity of the mitotic stages and the non-random distribution of breaks are further indications that the relationship between ionization and chromosome breakage cannot be reduced to simple mathematical formulae.

5. *Chromosome breakage and the role of oxygen*

There is other evidence which suggests that *direct* ionization is not the immediate cause of breakage. For example, the frequency of x-ray produced chromosome rearrangements depends on the concentration of oxygen present during irradiation. When the root of *Vicia* is exposed

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to x-rays in nitrogen, air or oxygen, the ratio of chromosome damage per cell in one experiment was 0·48, 1·0 and 1·75 respectively.[89] This observation suggests that ionization initiates a chemical reaction (formation of free radicals) in which the oxygen plays an important part.[90] Further investigation has revealed that the oxygen effect is exerted on the initial breakage mechanism.[91]

After these facts became known it was a logical development to analyse the effect of oxygen alone. CONGER and FAIRCHILD[92] exposed

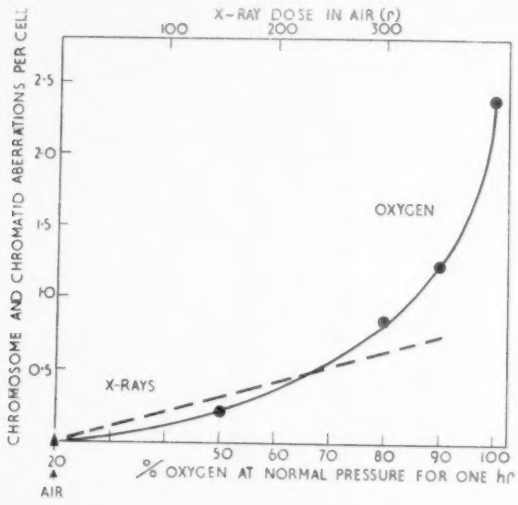

Fig. 36. Frequency of chromosome and chromatid breaks induced by oxygen concentration and x-rays. (After CONGER and FAIRCHILD[92])

dry pollen grains of *Tradescantia* to high oxygen concentration or pressure and found that treatment for one hour with 65 per cent oxygen concentration produced chromosome fragmentation equivalent to 306 roentgens (Fig. 36); 20 per cent oxygen (normal concentration in air) had no effect, though it may be responsible in some way for the 3 per cent residual "spontaneous" breaks found in the control series. The mechanism by which oxygen affects the chromosomes is very likely the same as that involved in radiation with x-rays.

6. *Protection from radiation injury*

If the product of oxidizing agents represents the initial step in the radiation reaction, then by destroying these agents it is possible to protect the sensitive reacting molecule. The effects of anoxia and hypoxia (complete or partial absence of oxygen) have been investigated and were found to reduce the yield of chromosome breakage. It was also shown that the LD50 had to be raised considerably when the irradiation of the

animals was carried out under conditions of hypoxia. These facts suggest that the primary products of ionization are oxidizing agents (free radicals) and that these agents are responsible for initiating the genetic changes such as gene mutation and chromosome breakage.

If the death of the organism following exposure to x-rays is due to gene or chromosome injuries and losses then it follows that the great variety of "protecting substances" which increase the LD50 should also reduce the frequency of chromosome breakage. But this is not always

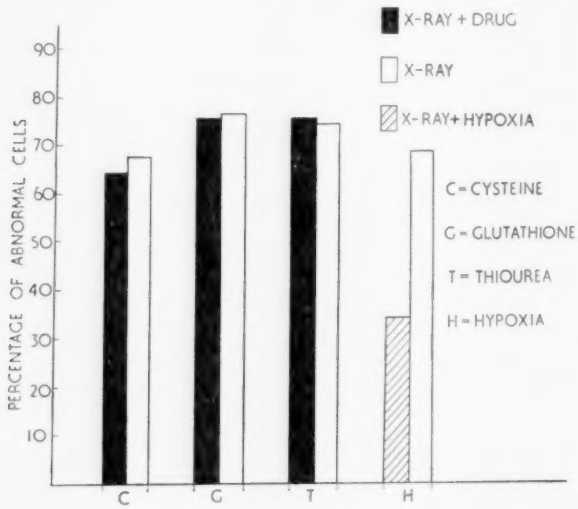

Fig. 37. The frequency of femoral marrow cells in mice with broken chromosomes 22 hours after 200 r with and without protective substances and hypoxia. (DEVIK[93])

the case. DEVIK[93] found that 200 r caused chromosome damage in about 70 per cent of cells in the femoral marrow of mice. There was no reduction in the number of cells with chromosome fragments when non-buffered cysteine hydrochloride, gluthathione and thiourea were administered shortly before irradiation. These substances are effective agents in protecting rats from radiation injuries. On the other hand, when the irradiation was given during exposure to severe oxygen deficiency, the proportion of abnormal mitoses decreased from 65 to 34 per cent (Fig. 37). When, however, neutralized cysteine was used, it had the same effect as hypoxia.[93a]

It is obvious that the effects which radiation produces within an organism are of different kinds and the overall injury is the outcome of both chromosomal and cytoplasmic disturbances.[94] When anoxia and cysteine are combined together, their protective effects become additive, yet the frequency of chromosome breakage may be the same as

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that found after radiation under conditions of anoxia alone. It can be assumed therefore that cysteine and similar protective agents interact with the cytoplasmic radiation effects, and may not affect the reaction which leads to chromosome breakage.

In order to throw light on the interaction between the various cell components (enzymes, chromosomes and genes) determining the response of the cell to external factors, our survey must enter into the realm of biochemistry. The first step in this direction is the study of chromosome behaviour in cells which have been exposed to chemical agents.

Chemically Induced Breakage

Since the discovery of mitosis, experiments have been carried out for the purpose of studying the effects of different chemical substances on cell division. As early as 1887 the Hertwig brothers[95] reported the induction of multipolar spindles and the suppression of mitosis by a great diversity of narcotics and alkaloids. Nemec[96] described in detail the effects of chloral hydrate on chromosomes. These and many similar experiments have shown up the immediate toxic effects induced but have failed to follow the behaviour of the affected cells after recovery.

When the mutagenic effect of x-rays on genes and chromosomes had been demonstrated, investigations were started to produce mutations by chemical agents.[97] The rediscovery of colchicine[98] and its application for doubling the number of chromosomes in plants[99], [100] gave a new impetus to the search for similar substances. The cytological effects of many drugs very often became known by necessity. We may mention the "fungicide" mercury ethyl phosphate as an illustration. After the seeds of maize had been soaked in this solution, injuries developed in the meristem cells causing the death of the seedlings. The loss was great and threatened maize breeding with serious financial consequences. Cytological analysis has shown that the mercury compound produces irreversible changes in the nucleus and chromosomes[101] and this finding led to the abandonment of the fungicide agent. Many observations of a similar nature were made; they are summarized by Sentein.[102] Since 1937 Levan and his co-workers have been making systematic studies of the toxic effects produced by several chemical substances in the chromosomes of *Allium* and they have reported many interesting observations. The drugs investigated, however, had no genetical significance; they induced either fatal chromosome injuries, or temporary cytoplasmic effects from which the cells recovered.

The first successful attempt to produce permanent structural changes in the chromosomes by chemical agents must be attributed to Oehlkers.[103] By using a mixture of ethylurethane and potassium chloride he obtained translocations in the meiotic chromosomes of

Oenothera and *Campanula*. Coinciding with his discovery were the positive results of AUERBACH and ROBSON[104] who found that mustard gas ($\beta\beta'$-dichloro-diethylsulphide) increased the mutation rate of sex-linked lethals in *Drosophila*, some of which by genetical tests indicated the presence of gross structural changes in the chromosomes.[105] At the same time cytological studies of mustard gas effects were undertaken by the author using the root-meristem of *Allium* and pollen grains of *Tradescantia* (Figs. 38–41). (Reports to Ministry of Supply 1943).[105a] Details describing the chromosome effects have been published later by DARLINGTON and KOLLER[106] and provided the basis of many similar observations. Some of the important findings may be summarized as follows:

(1) The centromere of meiotic and mitotic chromosomes is particularly sensitive to mustard gas; it can split transversly and undergoes misdivision.

(2) Breakage of chromatids is frequent at or near the centromere, the breaks usually being followed by chromatid interchanges.

(3) Chromosome stickiness often produces "pseudo-chiasmata"; the sticky anaphase bridges can break at any one level in the intercentric segment.

(4) The amount of fragmentation of chromosomes increases from 6 hours to 72 hours after treatment.

(5) Chiasma frequency i.e. the natural breakage in the meiotic prophase chromosomes, is increased.

By using the meristem cells of *Vicia*, FORD[107] studied the cytological effects of another chemical; methyl-bis-(β-chloroethyl)amine, known as nitrogen mustard (HN2). He found that

(1) Chromosome breakage appears later after HN2 treatment than after γ-radiation.

(2) Complementary deficiencies and duplications are due to structural changes involving sister chromatids.

(3) Equal chromatid interchanges are produced due to breaks at identical loci in the chromosomes.

(4) The distribution of breaks amongst the chromosomes is not at random. While the total length of the short (S) and long (M) chromosomes of *Vicia* shows a ratio of 5 : 2, the ratio of breakage frequency is 49·5 : 2 respectively.

(5) While the breaks after γ-radiation are equally distributed along the short chromosomes, after HN2 treatment they are "almost exclusively median in position." FORD concluded that HN2 has a selective action on particular chromosome regions, which he later identified as the heterochromatin blocks.[108]

The genetical and cytological effects of mustards are very similar to those produced by x-rays. Their pharmacological and toxicological

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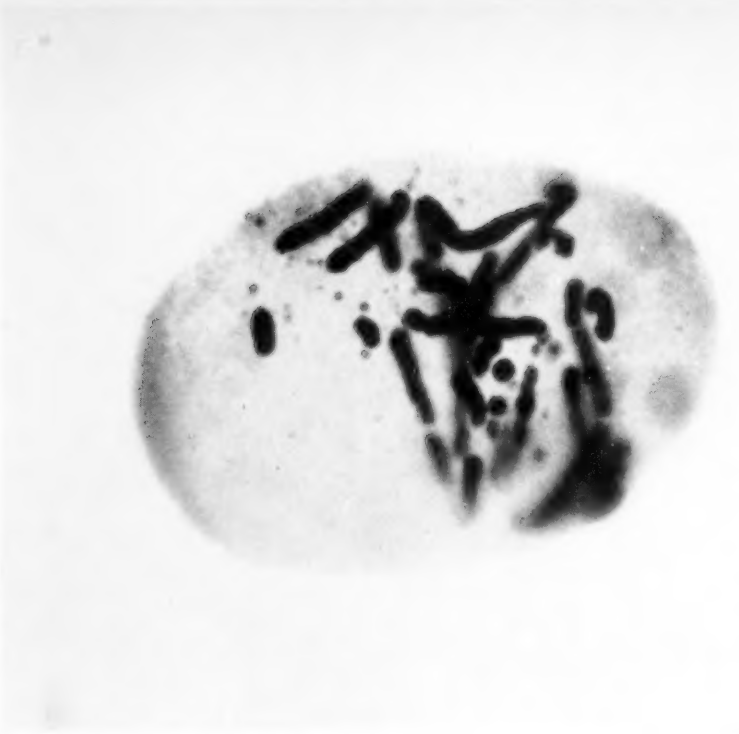

Fig. 38. Pollen grain of *Tradescantia* 24 hours after treatment with aerosol of sulphur mustard. The chromosomes have undergone extreme breakage (see Fig. 2). ×2000

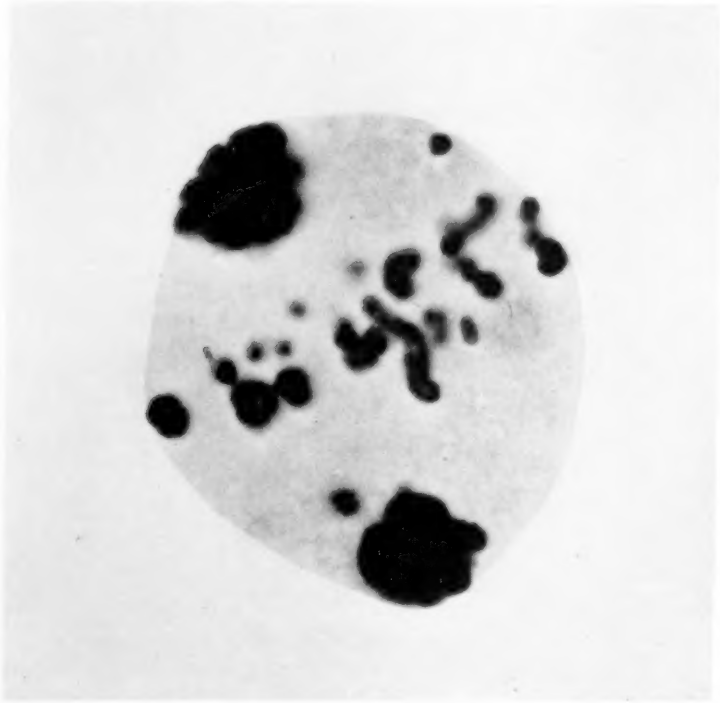

Fig. 39. Pollen mother cell of *Tradescantia* showing acentric fragments produced by mustard gas treatment. ×2000

Fig. 40. Pollen grain of *Tradescantia* with a lagging chromosome showing misdivision of the centromere (see Fig. 23). ×2000

Fig. 41. Chromosome breakage and chromatid interchange in the centric region induced by mustard gas treatment in the pollen grain of *Tradescantia*. ×2000

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properties have emphasized this similarity even more, and the mustards came to be known as chemical agents with *radiomimetic effects.*[109]

Substances with mutagenic and chromosome breaking ability are referred to in the literature as "chemical mutagens" to discriminate them from the physical mutagens (x-rays, ultraviolet). During the last few years the number of the former has been very much increased. Thus, acenaphthene, acetophenone, many acridine derivatives, bromine, morphine, naphthalene derivatives, purines, phenols, penicillin, putrescine etc. are all claimed to produce chromosome breaks either in plant or in animal material. They show a great diversity in molecular configuration

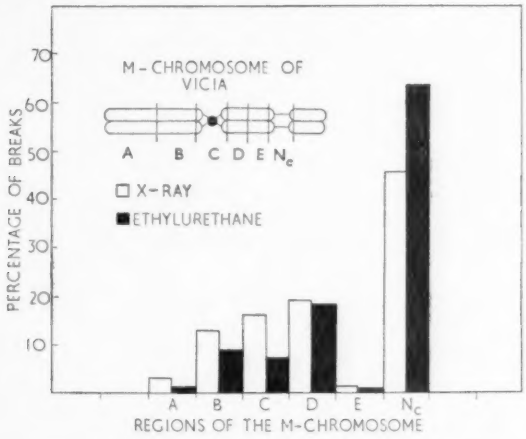

Fig. 42. Distribution of breaks in the various segments (A–B–C–D–E–N_c) of the M-chromosome (*Vicia*) after ethylurethane treatment (After DEUFEL[111])

and in effectiveness or potency. It is not the aim of the present article to discuss the merits of the claims, in view of the difficulty which naturally arises when the biological potency of these substances is compared. While some of the effects produced may be described as "radiomimetic," e.g., those produced by mustards or di-epoxides, many of the observed chromosome breaks are only primary injuries as is the case of phenols, pyrogallol or coumarin.[110]

The analysis of the cytological effects induced by the various chemical mutagens has already revealed many interesting features, some of which will be discussed below.

1. *Localization of breaks*

It has been already mentioned that in a few instances after x-rays more breaks were seen in particular chromosomes or chromosome regions than were expected on the basis of a random distribution.[87], [88] The localization of breaks is a much more common phenomenon after

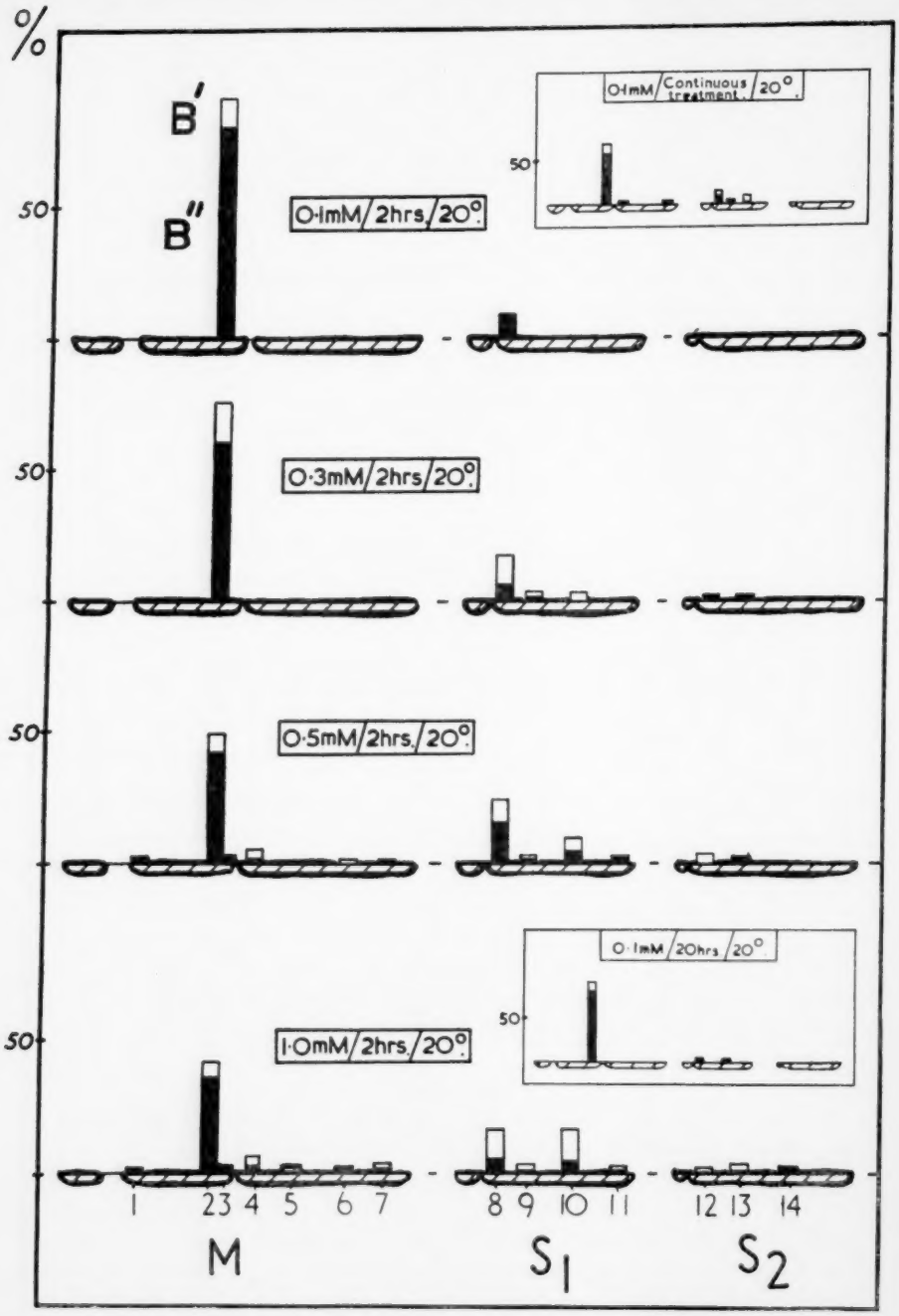

Fig. 43. Distribution of the heterochromatic breakage segments (numbered 1 to 14) in the 3 chromosome types M, S_1 and S_2 in *Vicia* after various maleic hydrazide treatments, with the total breaks in each. B' (clear) and B'' (black), the chromosomes are hatched. Insets contrast breakage distribution with the same mass action product (McLeish[112])

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chemical treatment. Different substances may select different chromosomes of the same plant. Thus nitrogen mustard produces more breaks in the *S* than in the *M* chromosome of *Vicia*.[107] (The distribution of breakage points in *Vicia* after HN2 treatment has been illustrated by GRAY in *Progress in Biophysics* 3 Fig. 4 page 264.) Ethylurethane and maleic hydrazide on the other hand produce more breaks in the *M* than in the *S* chromosome[111], [112] (Fig. 42). After treatment with pyrogallol, ethoxycaffeine, tetramethyluric acid and ethylurethane, the *M* chromosome breaks preferentially at the secondary constriction.[151]

The most extreme case of localization is reported by DARLINGTON and MCLEISH.[115] After maleic hydrazide treatment, the breaks in the *Vicia* chromosomes are restricted to 14 distinct segments, 8 of which correspond to the heterochromatic segments so far identified. The other 6 segments in which the breaks are concentrated are constant in position and may represent heterochromatin material too small in amount to detect (Fig. 43). Breaks induced by di-epoxy-propyl-ether and tertiary butyl alcohol tend to concentrate also in the heterochromatin blocks.[116]

What can be the cause of localization? There are two possibilities to consider. Firstly, the breaks occur in the heterochromatic segments because these regions by virtue of their peculiar nature react with the chemical agent more readily than the euchromatin. Secondly, it is possible that the breaks accumulate in the heterochromatin owing to its spatial arrangement within the nucleus. By studying living cells, FELL and HUGHES[4] found that the chromocentres, formed by the fusion of the heterochromatin blocks are attached to the nuclear membrane. (Fig. 44). The heterochromatin in this position is an easy target for attack.

The importance of the spatial distribution of chromosomes within the resting nucleus has been already pointed out by FORD and was emphasized by OEHLKERS *et al.*[117], [118] who found that certain chromosomes of *Oenothera* are involved more frequently than others in forming interchanges. Owing to the fusion of the heterochromatin segments breakage is favoured at identical loci in the homologous chromosomes and results in symmetrical rearrangements.[116]

2. *Delayed effects*

It was noticed by AUERBACH[119] that the number of *Drosophila* with mosaic patches in the body increased after mustard gas treatment as compared with X-rays. Such flies are the results of chromosome elimination which occurs during the cleavage division. The size of the mosaic area in the body indicates the development stage at which this event took place. After X-rays it always occurs at the first cleavage division, and half of the adult fly is affected. After mustard gas treat-

ment mosaics were obtained in which the area covered less than half of the body indicating that chromosome elimination took place in the second or later cleavage division. The most important evidence for delayed effect is the reappearance of mosaic offspring from a parent which had mosaic patterns in the body including the gonads. It is very probable that the treatment induced a labile state in the centromere which under certain physiological conditions leads to inactivation. The

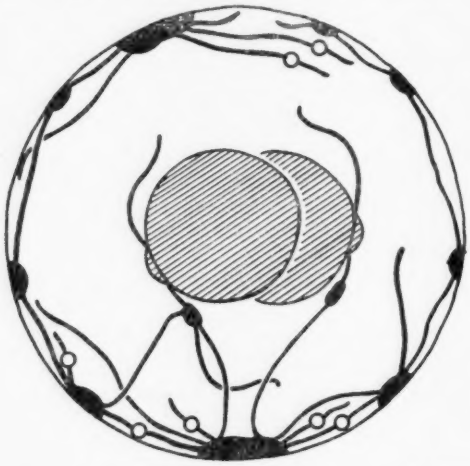

Fig. 44. Diagram showing how the chromosomes of *Vicia* may be anchored in the resting nucleus by the *chromocentres* (black) and nucleoli (hatched). Non-random fusion of chromocentres causes non-random reunion. (McLeish[112])

great sensivity of the centromere to chemical agents has been already pointed out[106] and it may be responsible for the high incidence of chromosome elimination.

Another instance of delayed effect is the great increase in the frequency of "somatic crossing over," which was studied in *Drosophila*. The visible results of this event are the "twin spots" on the body of the adult fly. In view of the difficulty in recognizing such small spots, their high incidence shows that "somatic crossing over" is a very common event after HN2 treatment.

3. *Partial breakage*

Certain types of aberrations were seen after x-ray and ultraviolet radiations in the chromosomes of the pollen tube nucleus of *Tradescantia*, which can be interpreted as "half-chromatid" breaks and reunions[120]. Similar observations were made in *Bellevallia*[121], [122] and in *Uvularia* after x-radiation.[123] Such "incomplete" breaks were also found in *Paeonia* after ethylurethane treatment.[124] This aberration raises the important questions: What is the functional and elementary unit of

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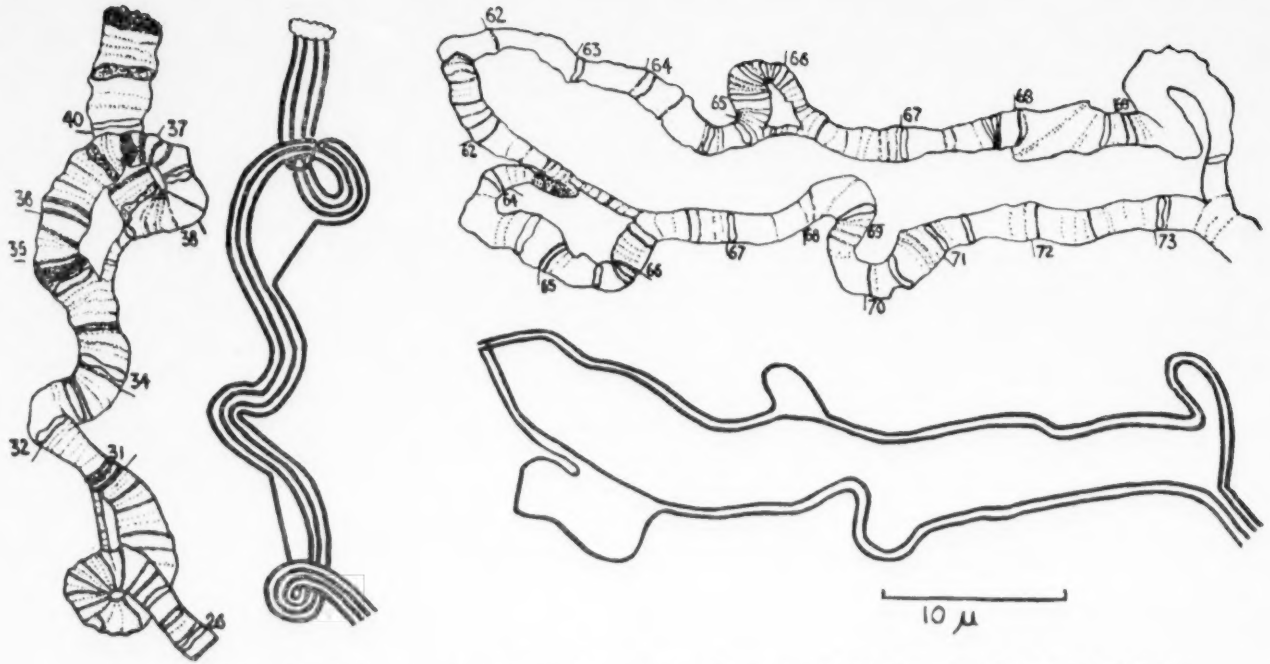
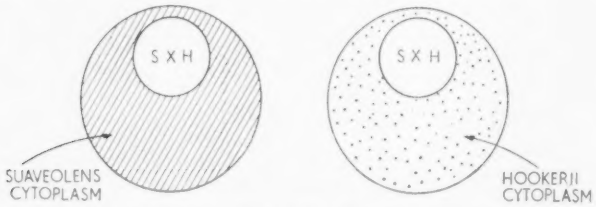


Fig. 45. Salivary gland chromosomes of *Drosophila* showing deficiencies in the subunits. 13 hours old embryo, irradiated with 500 r. (SLIZYNSKI[125])

the chromosome body? Is the chromosome of a mitotic chromosome composed of several, genetically identical filaments, like the polytene chromosomes of the salivary glands? Structural changes which involve less chromosome material than a chromatid, have been seen in the polytene chromosomes of *Drosophila*, after irradiating larvae which were only a few hours old,[125] (Fig. 45) and similar incomplete breaks were also found in these chromosomes after di-epoxy butane treatment.[126] It is very probable that the chromosome thread of mitosis is made up of a bundle of similar polypeptide chains;[126a], [126b] x-rays and chemical agents in certain instances may fail to break all the sub-units.

4. *Variation in response*

While the chromosomes of *Vicia* can be broken by a 30 minute treatment with 10^{-5} molar solution of the nitrogen mustard, a very much



	Percentage of Broken Chromosomes	
x-ray . . .	10·2	10·6
Ethylurethane	7·3	10·7

Fig. 46. Diagram illustrating the breakage frequency of chromosomes in the reciprocal crosses of *Oenothera suaveolens* × *Hookeri* after 150 r x-ray and ethylurethane treatment. (From OEHLKERS[117])

higher concentration is required to produce breaks in the chromosomes of *Allium*[127]. Various purine derivates of the same concentration produce qualitatively as well as quantitatively different cytological effects in *Pisum* and *Allium*.[128] OEHLKERS[117] obtained different breakage frequencies in reciprocal crosses of *Oenothera* after ethylurethane treatment but not after irradiation (Fig. 46). The difference must be attributed to the cytoplasm which alone differs in the reciprocal crosses. D'AMATO[129] found that in *Allium* the same concentration of acridine produced a significantly different breakage frequency in the spring and autumn, which may be attributed to the different growth rate during these seasons. The rate of absorption is dependent on the rate of growth, consequently the chromosomes of a slow growing plant show fewer breaks, or the breaks appear much later, than in plants with

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higher rates of growth, after treatment with the same drug in the same concentration.

It seems that so far only the heterochromatin of *Vicia* chromosomes can respond to maleic hydrazide; other plants with large amounts of heterochromatin in their chromosomes, e.g. *Muscari, Scilla* and *Rhoeo* are immune to this drug.[115] Similarly no chromosome breaks were found in animals' cells after maleic hydrazide treatment.

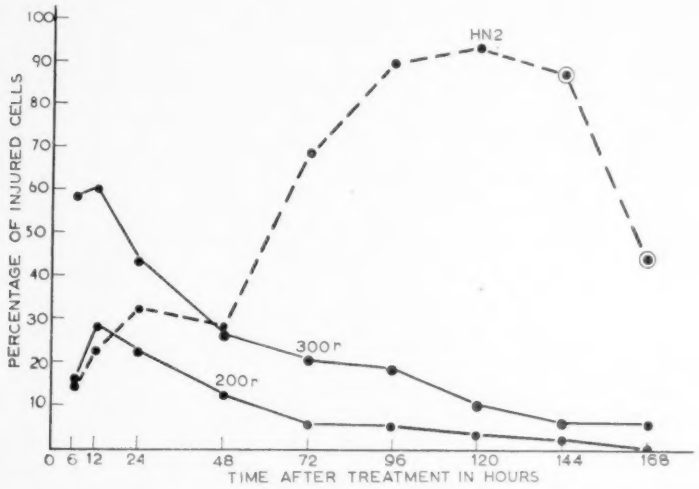

Fig. 47. Graph showing the percentage of cells with chromosome fragments in a rat tumour after x-ray and nitrogen mustard (HN2) treatment. (After KOLLER and CASARINI[130])

The few examples described, show that the action of the chemical mutagens depends on and is influenced by genetical and environmental factors. This fact should warn us against generalization.

5. *Period of sensitivity*

The measure of sensitivity of a cell is the frequency of chromosome injuries observed in a given time. The interval between treatment and fixing the cell for analysis is believed to indicate the stage in which the cell was treated. The reliability of this method is determined by various conditions which must be fulfilled, e.g., (1) the transportation of the drug to the site of action should be rapid; (2) its activity should be of short duration within the cell (a few minutes); (3) the rate of cell development should not be altered by the drug, etc. Cytological studies suggest that nitrogen mustard fulfils these conditions, and it was found both in *Vicia*[107] and in a transplanted rat tumour[130] that the cells are most sensitive to this drug at the beginning of resting stage (Fig. 47).

By employing a double treatment technique, REVELL[116] demonstrated more accurately the time of greatest sensitivity during cell development for x-rays and di-epoxy-propyl-ether (Fig. 48). The data leave no doubt that the cells are most sensitive to chemical agents so far studied, at the very beginning of resting stage, and are most sensitive to x-rays at the end of the resting stage. This fact is of primary importance for interpreting the mode of action of the physical and chemical mutagens.

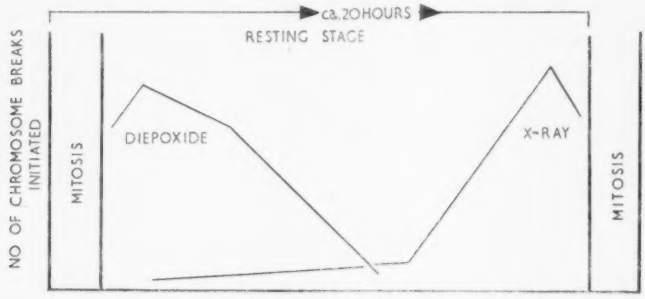

Fig. 48. The sensitivity of resting stage in *Vicia* to di-epoxy-propyl-ether and x-ray as measured by the number of chromosome breaks initiated. (After REVELL[116])

6. *Molecular structure and destructive potency*

After the discovery of sulphur mustard as a chemical mutagen, many compounds of similar structure were synthesized and tested. It soon became established that the mutagenic activity might be connected with a special molecular configuration. Thus, in the nitrogen mustard and mustard-like compounds the presence of two alkylating groups was believed to be essential to produce chromosome damage. When monofunctional and bifunctional alkylating agents are tested for their activity to break chromosomes, concentrations about 50 times greater are required in the former case compared with the latter.[131]

D'AMATO found that when CH_3O and Cl are substituted for the amino groups in the acridine molecule, it became inactive.[129] The potency of 25 purine derivatives was compared by KIHLMAN[128] who found that 8-substituted caffeine derivatives are the most effective compounds. Xanthine and uric acid are inactive. It was also observed that the potency of oxypurines as chromosome breakers increases with increasing N-methylation, while that of the methylated oxy-purines decreases or is lost, if methyl-groups are substituted with groups possessing longer carbon chains. KIHLMAN concluded that as regards the purines, chromosome breaking ability does not depend upon any particular atom or groups of atoms, but on the molecule as a whole.

The cytological effects of 29 tetra-sodium-hydroquinone diphosphate analogues were compared by MITCHELL and SIMON-REUSS.[132] They

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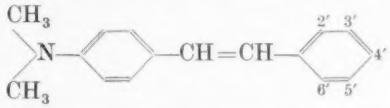
found that the presence of phosphate groups is essential for inhibiting mitosis, and the presence of methyl-groups is necessary for the production of chromosome breaks.

The chromosome breaking ability of the aminostilbenes has been analysed by the author. These compounds were found to inhibit the growth of the Walker carcinoma in varying degree.[133] Inhibition of the tumour was found to be due to degeneration of mitotic cells which is brought about by chromosome injuries. The molecular structure of the parent compound (4-dimethylaminostilbene) is



The frequency of dividing tumour cells showing chromosome fragmentation in the anaphase stage was the criterion by which the efficiency of the various substitutions was determined and compared.

Table 3

Comparison of the cytological effects of three derivatives of 4-dimethyl aminostilbene (Walker carcinoma in the rat; 48 hours after intraperitoneal injection).

Substitution		*Dose* (mg/Kg)	*Percentage of anaphases with chromosome fragments*
Nature	*Position*		
Chloro	2′	50 mg	44·8
	3′	50 mg	34·8
	4′	50 mg	9·3
	4′	125 mg	18·0
	4′	250 mg	32·8
Methyl	2′	50 mg	26·8
	3′	50 mg	28·2
	4′	50 mg	10·0
	4′	125 mg	21·0
	4′	250 mg	20·0
	4′	500 mg	24·0
Fluoro	4′	50 mg	52·3
	4′	100 mg	78·3

Table 3 shows the results obtained 48 hours after intraperitoneal injections of the various aminostilbene derivatives. It seems that the compound substituted in the 2′ or *ortho*-position is about 10 times as effective as that substituted in the 4′ or *para*-position. On the other hand the high degree of chromosome breaking ability of 4′-fluoro-substituted aminostilbene indicates that beside the position, the nature

of the substitution is another important factor which determines the amount of biological damage. When the effects of the various analogues were compared 24, 48 and 72 hours after treatment, another interesting feature of their activity was brought to light (Fig. 49).

The chromosome breaking ability of seven di-epoxide analogues has also been studied using the same method. Some of these compounds

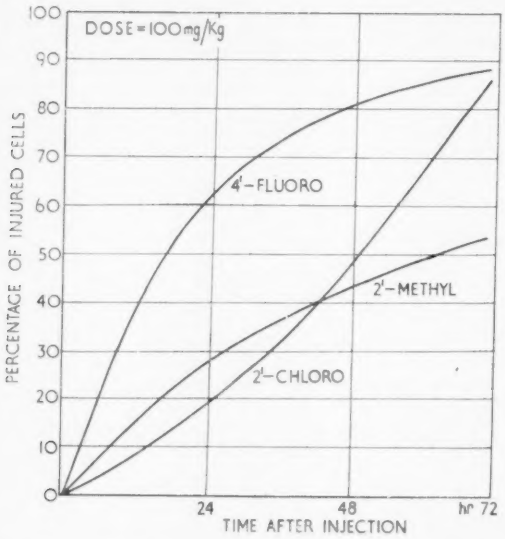


Fig. 49. Graphs showing the percentage of injured tumour cells in rat after the intraperitoneal injection of the same dose of three aminostilbene derivatives.

were reported to induce chromosome breaks in *Vicia*,[110] to increase the mutation rate of sex-linked lethals in *Drosophila*,[126] and to inhibit the growth of the Walker carcinoma in the rat[134], [135]. The basic formula is

$$CH_2\text{—}CH\text{—}(CH_2)_n\text{—}CH\text{—}CH_2$$
$$\quad\ \backslash O /\qquad\qquad\quad \backslash O /$$

and the seven compounds analysed differed in the number of carbon atoms in the methylene bridge. The criterion of their efficiency was the dose required to produce about 30 per cent of abnormal anaphases in the Walker tumour 48 hours after intraperitoneal injection. Fig. 50 shows the data obtained. The destructive potency decreases with the increase in the number of carbon atoms, i.e. the greater the distance between the two epoxy groups of carbon atoms, the less active is the compound.

While the di-epoxide series indicates a simple relationship between

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molecular structure and activity, the behaviour of the aminostilbenes shows how difficult it may be to relate activity to molecular structure.

7. *Mode of action*

In almost every report claiming the discovery of a new chemical mutagen, the fortunate author attempts to explain the mechanism by which the biological effect is brought about. The chemically induced genetical and cytological effects seem to be one of the most inviting topics of speculation, in which cytologists, geneticists and chemists share equally. There is a general tendency to find one common

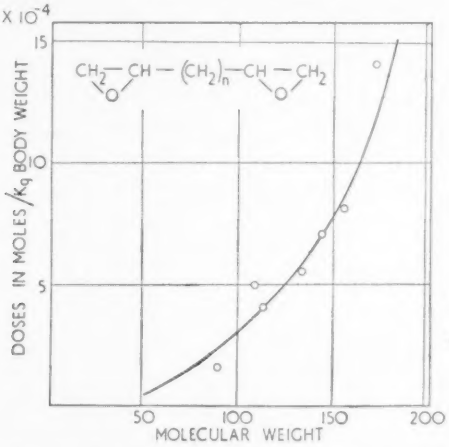

Fig. 50. Graph showing the relationship between molecular weight (number of carbon atoms) and biological potency of seven di-epoxide analogues.

mechanism by which the chromosomes respond to various chemical agents. The present position closely resembles that which occurred when great effort was spent in applying the "target-theory" of gene mutation to the complex phenomenon of chromosome breakage.

In spite of the similarity shown by the end-effects of chemical mutagens, it can be assumed *a priori* that the mechanism by which these effects are produced, is not the same. On what basis can it be expected that compounds such as the mono- and bi-functional mustards, ethyl-urethane, maleic hydrazide, purine derivatives and the various amino-stilbenes should have a uniform mode of action within the cell?

When considering the mechanism by which chemical agents affect the chromosomes, two important assumptions are implied. First, that the drug has entered, or has been absorbed by the cell without being altered during this process; secondly that the drug attacks the chromosomes directly. The first assumption may be justified in experiments in which the meristem cells of growing root-tip are brought into contact

with the aqueous solution of the compound under test. When, however, the drug is injected or fed, or when it is used as an aerosol to which the whole organism has been exposed, the cytological effects under study may be the products of greatly different metabolites.

With regard to the second assumption, too little is known at present about the site of action of chemical mutagens within the cell. Such information may be obtained by cytochemical methods, or from studies in which the chemical mutagens are labelled by radioactive elements. Another technique, in which the cell nucleus and cytoplasm can be exchanged, may also be used with profit. By employing the latter method DANIELLI and co-workers[136] investigated the biological effects of mustard in the *Amoeba*; they found no evidence of a localized attack and concluded that the action of HN2 in the cell is "diffuse," There was no selectivity for either nucleus or cytoplasm.

It has has been already emphasized that the similarity of end-effects produced by different agents is not sufficient evidence to postulate a similarity in the primary mechanism. This was clearly illustrated by comparing the cytological and genetical effects of x-ray and nitrogen mustards. The latter imitates x-rays in many respects; it is a "radiomimetic" poison. Yet it has been shown that the mechanism by which nitrogen mustard breaks the chromosomes must be different from that involved in producing the effects of ionizing radiations. While the frequency of sex-linked lethal mutations in *Drosophila* induced by radiation is much reduced when the irradiation is carried out in the absence of oxygen, no such reduction was observed when nitrogen mustard is used as the mutagenic agent.[137] In the former, oxygen plays an important role, but it is not essential for the production of nitrogen mustard effects.

Various hypotheses have been formulated and put forward to explain the mode of action of groups of substances which are similar in molecular structure. Thus, it was suggested that the mustards act by inactivating or poisoning an —SH group either in the proteins of the chromosomes or in the phosphate transferring enzymes. When the importance of two alkylating groups in the mustard molecule had been demonstrated, it led to another hypothesis which assumed that the mustards act by forming "cross-linkages" between neighbouring fibres of chromosomes.[110] The cross-linkage hypothesis was applied to other bifunctional compounds such as the di-epoxides and ethyleneimines. There is however much evidence to show that the possession of two biologically active groups and the ability to form cross-linkages between protein chains are neither indispensable nor universal properties of the chemical mutagens.[138–140]

The cross-linkage hypothesis assumes the attack to be a direct one on the chromosomes. While it may be so in the reactions initiated by

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mustards and di-epoxides, in the case of ethylurethane no direct attack can be postulated. The importance of the cytoplasmic enzyme systems in the ethylurethane induced effects has been shown by OEHLKERS[117] who found significant differences in breakage frequency in reciprocal crosses of *Oenothera*.

The ability of a drug to penetrate the cell and nuclear membrane, the rate of its absorption by the cell and the solubility of the agent are important physical properties which influence and very often condition the biological activity. A good example is provided by the purine analogues. In respect of their mode of action they fall into two groups: the first is represented by 8-ethoxycaffeine, the second by 1 : 3 : 7 : 9-tetramethyl uric acid. The analogues of the first group can penetrate the nuclear membrane and act before cell division, while those which belong to the second group cannot penetrate and act only after the cell has undergone mitosis.[128]

In the benzene and naphthalene groups of substances with colchicine-like effects[141] close correlation is claimed to exist between activity threshold and water solubility. Similar correlation is reported for the phenols and related substances.[142] The reduced biological potency of those di-epoxides which have a large number of carbon atoms may be due to a reduced rate of absorption.

When the action of the chemical mutagens within the cell is interpreted, the biochemical aspect of chromosome synthesis and the complexity of intracellular interactions between cytoplasm and nucleus must be considered. The chromosomes are dynamic entities within the cell and "it is becoming increasingly evident indeed, that the chromosomes are not static units with genes lined up like cars in a railroad train."[143] Their structure is not permanent, it is continuously rebuilt from the material of the surrounding medium.[143a] The chemical mutagens not only attack the chromosomes already visible but they also interfere with the process of chromosome synthesis. By considering these aspects, the phenomenon of chromosome breakage takes up a greater significance; it becomes an essential part of the general biochemical mechanism which underlies cell reaction, adaptation and selection, i.e., evolution.

8. *Mitotic poisons and radiomimetic substances*

Chemical mutagens, like x-rays, injure the chromosomes in the resting stage nucleus and cause the death of the cells when they subsequently undergo mitosis. But cells which are in mitosis during treatment can also show abnormal chromosome behaviour due to physiological disturbances, i.e. cytoplasmic enzyme poisoning. According to the stage at which the cell is affected, the chemical agents are divided into two groups. Substances which cause the breakdown of cells in mitosis are

labelled "mitotic poisons" after DUSTIN.[98] They are distinguished from the "radiomimetic poisons" which affect the cells during the resting stage.[109] The latter are effective in rather low concentration and the relationship between concentration and mutation frequency is similar to that seen after ionizing radiation[145] (Fig. 51). If there is any primary or physiological upset, the cell recovers from it. It is possible however to produce toxic reactions which can be fatal to both dividing and non-dividing cells by increasing the concentration or duration of treatment of the radiomimetic agents. On the other hand, many

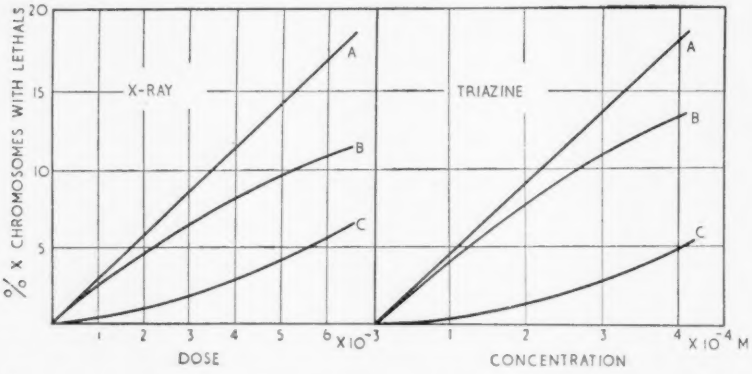

Fig. 51. Graph illustrating the dose/effect relationship between the various genetical changes in the chromosomes of *Drosophila* after x-ray and triazine [2 : 4 : 6 :-Tri-(ethylene-imino)-1 : 3 : 5-triazine]. (*A*) total lethal changes, (*B*) lethals without visible rearrangements in the salivary gland chromosomes, (*C*) lethals with rearrangements. Dose-effect relationship is the same for x-rays and triazine. (FAHMY and BIRD[145])

mitotic poisons which affect the cytoplasmic enzyme systems can indirectly cause chromosome fragmentation. Thus under certain conditions no distinction can be made between the two groups.

It is therefore proposed to classify not the agent but the cytological effects themselves. They can be divided into two types: (*i*) nucleotoxic and (*ii*) cytotoxic effects. The radiomimetic effects (chromosome breaks and reunion) fall into the first class, the physiological or primary effects (suspension of mitosis, clumping of metaphase chromosomes, super-fragmentation of chromosomes, nuclear pycnosis etc.) clearly belong to the second group. Cell degeneration, which is produced by an agent during resting stage, is considered to be a cytotoxic effect. The various types of these effects are shown in Fig. 52.

In the therapy of cancer, the aim is the destruction and elimination of the dividing cells responsible for the malignant growth. Whether the dividing cells die by chromosome injuries or by cytotoxic disturbances is of secondary importance (Figs. 53, 54, 55 and 56). The cytotoxic

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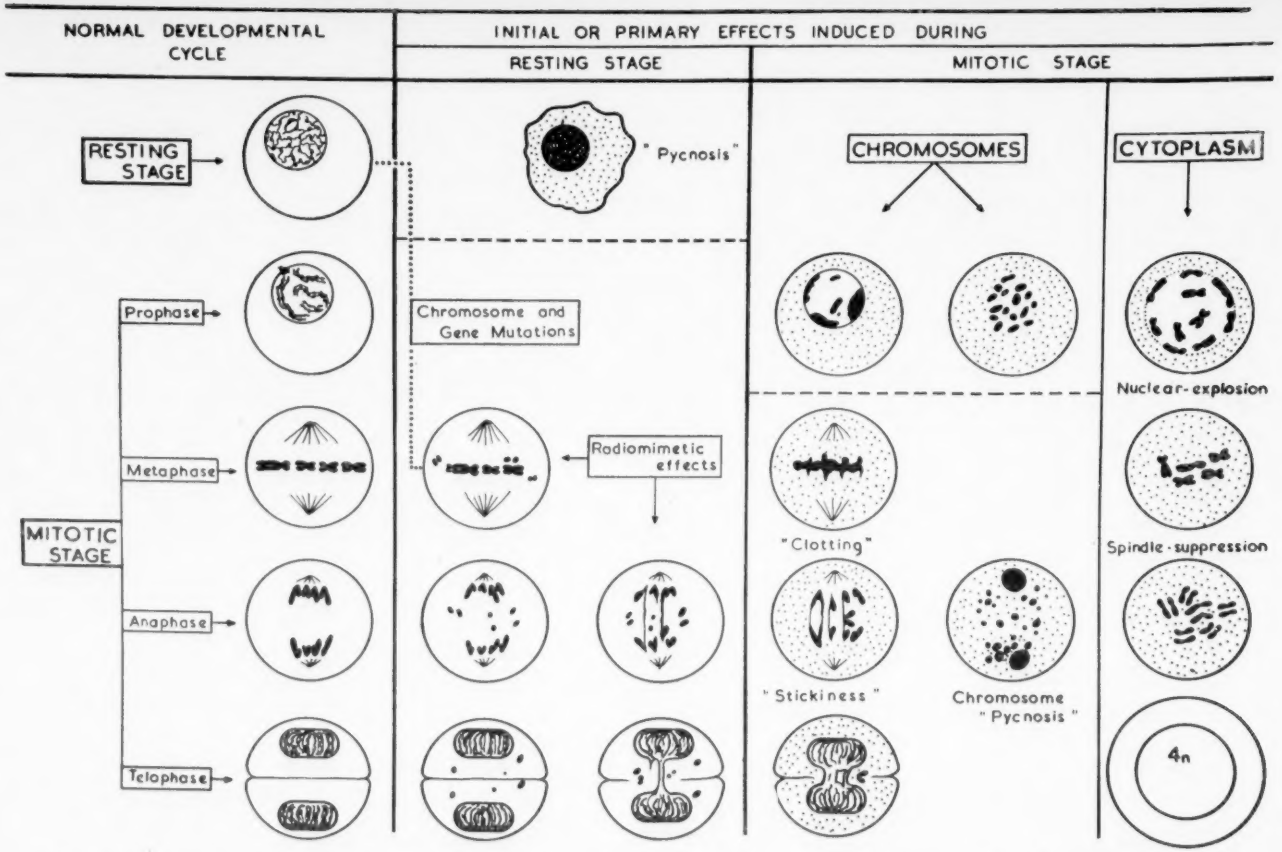

Fig. 52. Diagram showing the various types of cytological effects induced by mitotic poisons. Excessive dose or prolonged treatment can cause degeneration (pycnosis) during resting stage. Spindle suppression during mitos is leads to the doubling of chromosome number.

effects under certain conditions can be even more important than the true radiomimetic effects. Thus nitrogen mustard is a typical radiomimetic poison; it produces gene mutation and chromosome breakage in many animals and plants. Yet the therapeutic value of HN2 in lymphoid leukaemia and lymphosarcoma is not necessarily and wholly due to the radiomimetic properties. The cytological abnormalities observed in these particular species of cells after nitrogen mustard treatment indicate cytotoxic reaction.

While the highly specific radiomimetic substances may throw light on the vital process of chromosome synthesis and structural organization, they cannot be claimed to be the only class of mitotic poisons which have a potential value in the chemotherapy of malignant disease. These agents interfere with particular substances or their synthesis in the cell, which are precursors of mitosis. These substances are the same *in every cell* which is preparing for division. Thus the cells of the haemopoietic bone marrow, of the germinal layer in the testis, of the crypts of Lieberkühn in the intestine, will be attacked indiscriminately by radiomimetic agents introduced into an organism. The most serious shortcoming of these groups of substances is that they do not distinguish between the cells of normal and malignant tissues.

The chemotherapeutic value of a substance is determined by its ability to interfere with specific cell function by its selectivity, toxicity, absorption, permeability and duration of action.[146] There are very few ways by which the action of the radiomimetic agents may be restricted and localized in a particular cell or tissue. One possibility is to make use of specific enzyme systems which determine the character of cells and tissues. The effect of narcotics on nerve cells, or that of stilboestrol on the malignant cells of the prostate gland, is very likely brought about by this process. Myleran (1 : 4-di-methanesulphonyloxybutane) affects primarily the granulocytes in the marrow, and because of this selectivity became an important drug in the treatment of myeloid leukaemia.[147] As long as the cell has the morphological and chemical properties which characterize it as a member of a particular differentiated tissue, it responds to a particular drug. "Dedifferentiation" i.e. the loss of a characteristic enzyme complex, is a common, perhaps an unavoidable, occurrence in malignant cells (anaplasia). With the loss of this cell component the selective property of the drug becomes ineffective. If however the selectivity of the substance depends on several cell variables then its action is safeguarded to a certain degree from such eventualities.

Another way by which the action of chemical agents would be restricted to malignant cells or tissues would be to make use of some specific property common to all malignant cells. Thus the difference in *p*H between normal and tumour cells might be used for this purpose.

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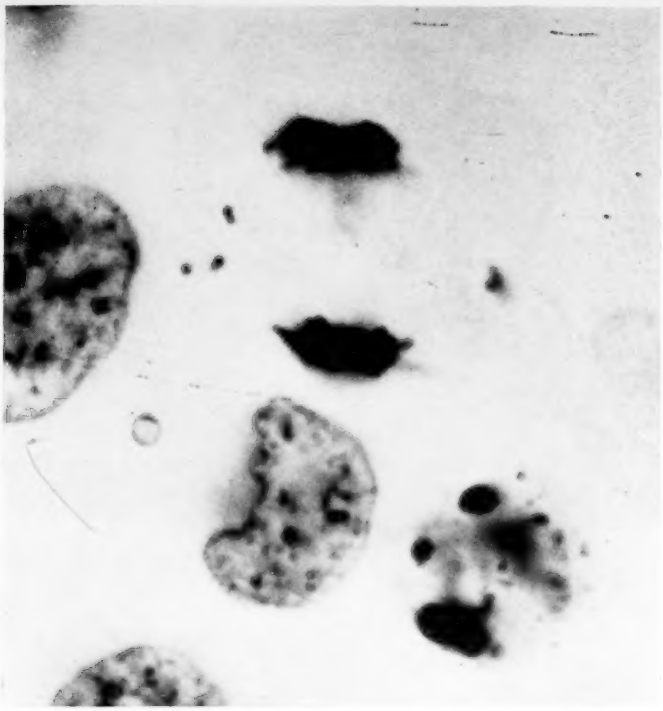

Fig. 53. Dividing cells in the transplantable Walker carcinoma of the rat 48 hours after intraperitoneal injection of 2′-chloro-4-dimethylaminostilbene. Cell at the top shows radiomimetic effects, in the cell at the bottom the mitotic mechanism is broken down indicating cytotoxic disturbance. × 1800

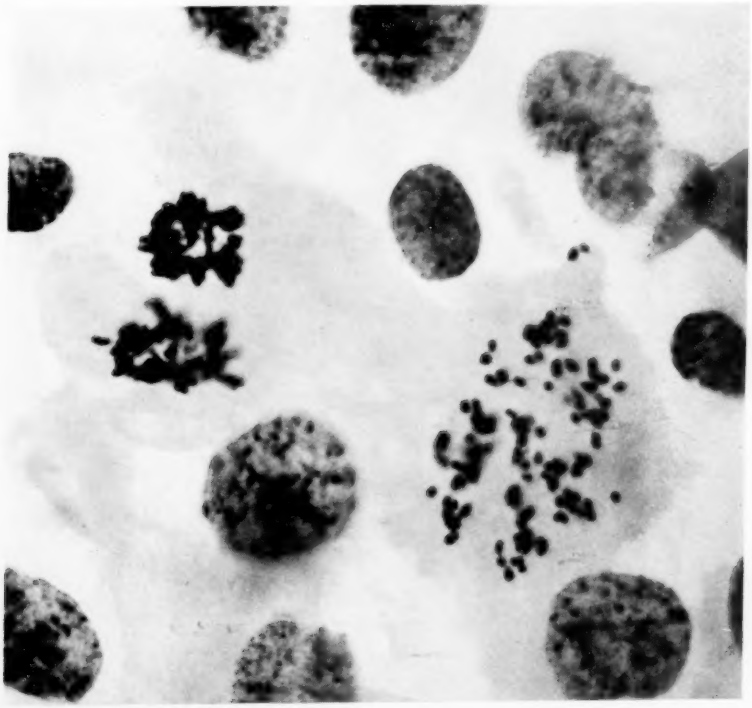

Fig. 54. Supercontraction of chromosomes and absence of mitotic spindle in a cell of Walker carcinoma of the rat 48 hours after 3′-methyl-4-dimethylaminostilbene. Cell on the left is undergoing normal anaphase. × 1800

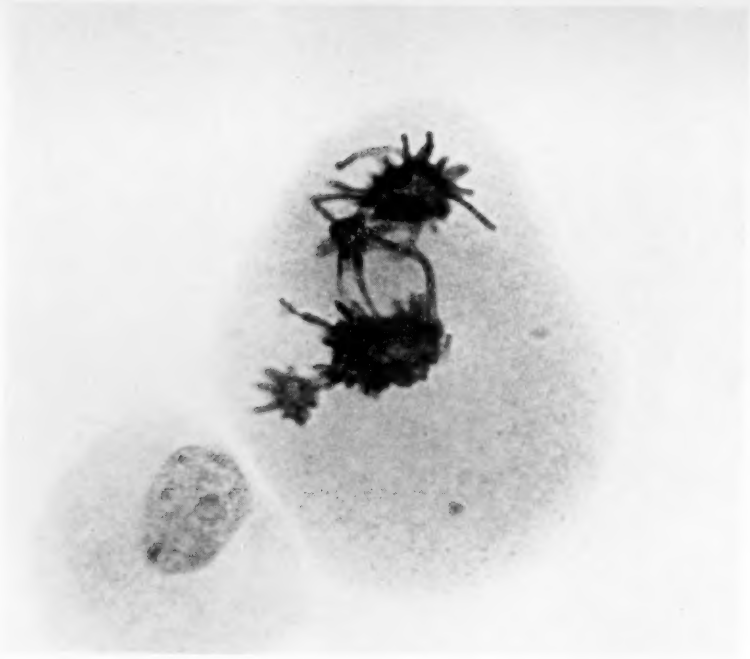

Fig. 55. Dicentric chromosome bridges (nucleotoxic effect) and multipolar spindle formation (cytotoxic effect) in a dividing ascites cell of Krebs carcinoma, 72 hours after treatment with an aromatic nitrogen mustard. × 1800

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Fig. 56. Superfragmentation of chromosomes in a tumour cell indicating an extreme degree of cytotoxic effect 48 hours after treatment with diepoxybutane. × 1800

Unfortunately, up to date no chemical property, common to all tumour cells, has been found.[148], [149] This fact shows the magnitude of the problem which confronts biologists and biochemists in their search for *selective* growth inhibitory substances. It is obvious from the foregoing discussion that the search cannot be restricted only to substances with radiomimetic effects, it should also be directed towards the larger groups of mitotic poisons with cytotoxic effects. The modern approach of the organic chemist to chemotherapy is governed by the idea that cells can be damaged by selective toxicity of a poison acting on those enzymes, which are essentials for intermediate metabolism in the particular cell-type.[150] The cytotoxic effects are the visible indicators of the induced biochemical lesions and reveal the enzyme disturbances, or enzyme inactivation within the cell under attack. They are important criteria by which the potential value of a substance in the chemotherapy of cancer may be judged.

Conclusion

The brief survey presented above, has described various types of chromosome breakage and has demonstrated the diverse agents which can induce injuries. We have seen that from the cytological aspects the physical and chemical mutagens break the chromosome thread apparently in a similar manner. The similarity between the chemically and radiation induced chromosome breakage is further emphasized by the rapid progress which has been made since 1945 in the study of chromosome behaviour under the influence of chemical agents and by the more recent advances in radiochemistry which have brought to light many important new facts.

In the present article the analysis has been extended to the internally controlled events of crossing-over, centromere fragmentation, dicentric chromosomes and "spontaneous" or natural breakage and evidence has been given that these are conditioned by the definite physiological environment within the cell. Radiation and the chemical mutagens might be considered to be initiators of similar physiological processes of which the breakage of chromosomes is a necessary consequence. Our survey leads us to the conclusion that in order to formulate a modern concept of the mechanism of chromosome breakage, the cell reaction to ionizing radiation and chemical agents must be viewed together with the phenomena of natural breakage, and other internally conditioned events which result in the disruption of the chromosome structure.

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THE CRYSTALLINE PROTEINS: RECENT X-RAY STUDIES AND STRUCTURAL HYPOTHESES

J. C. Kendrew

It is some time since x-ray studies of crystalline proteins were reviewed:[1], [2] considerable progress has been made since then, and now seems an appropriate moment to attempt a new assessment. There are two aspects in terms of which such an assessment may be made: first, that there have been developments of method, and second, that there appears to be a need for some evaluation to be made of the reliability of the results and of their relevance in more general fields of biology.

In the latter connexion there is this to be said by way of preamble. A major difficulty about what might be called the public relations of protein crystallography is that, on the one hand, the methods used are familiar only to crystallographers and not in general to biologists, who are therefore in a poor position to assess the reliability of the results claimed; and that, on the other hand, nearly all these results, however painfully arrived at, are provisional and incomplete. The protein crystallographer is faced with the dilemma, that either his results will be mis-interpreted, generally over-interpreted, by all those outside his own field; or that, if he hedges them about with qualifications designed to prevent this happening, they may appear to be no results at all—which is far from being the case. An analogous situation exists in some related fields; it is probably fair to say that the crystallographic results thus far should be taken no more and no less literally than, for example, the equivalent ellipsoids or the effective dipoles of the physical chemist. The truth is that up to now the results achieved of direct value to those outside the field are meagre: but on the other hand much progress has been made towards constructing a methodology and a self-consistent scheme of hypothesis which can yield important results of very direct relevance to structural biology within a reasonable time. In the meantime it could perhaps be argued that protein crystallographers should work in isolation without too much enquiry from outside. However such isolation would in the long run be unhealthy, and in any case the gratifying interest of biologists at large cannot and should not be discouraged. It will be part of the purpose of this review to attempt to satisfy this interest without painting an unduly optimistic picture of the present state of affairs.

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The next Section will review recent developments in methods of analysis; the third will briefly summarize the results hitherto obtained, as they might be regarded by those outside the field. Throughout the review discussion is confined to the crystalline proteins (excluding viruses). It is unfortunately not possible in an article of this kind to include the basic crystallographic theory necessary to make all the methods described fully intelligible to those not familiar with the field; nor has this theory been collected in any compact form elsewhere. However, efforts have been made to present the discussion of methods in the simplest possible way, and to describe the conclusions which have been drawn without recourse to crystallographic jargon. Consequently it is hoped that the passages unintelligible to the general reader are limited in extent, and will not prevent an understanding of the main trend of the discussion.

Methods of Analysis

Determination of cell constants and symmetries of crystalline proteins

The list of proteins whose crystals have been studied by means of x-ray diffraction is now a long one; the present situation is summarized in the Table at the end of this article. It must be emphasized that the information contained in this Table—information which may take only a day to obtain once crystals are available—is in itself of little value from the structural point of view. Almost all that can be derived from these basic crystallographic data is a value of the molecular weight of the protein, provided measurements have also been made of crystal density and composition (as is well known, protein crystals generally contain up to about 50 per cent of water or salt solution); such determinations have been made, for example, for ribonuclease[3] and for serum albumin.[4]

In some special instances the existence of repeating units *within* the molecule can be simply demonstrated. Occasionally it is found that a protein (whose molecular weight has been determined by independent means) occupies more than one asymmetric unit in the lattice (the asymmetric unit is the smallest part of the unit cell which, when operated on by the symmetry elements of the space group, yields the total contents of the cell). It then follows that the molecule must be subdivided into identical units, each corresponding to one asymmetric unit, and related by the appropriate symmetry elements. Thus horse haemoglobin normally crystallizes in the space group $C2$, whose unit cell comprises four asymmetric units, related in pairs by dyad axes; but in this crystal form the unit cell actually contains only two haemoglobin molecules. Hence the molecules must each be subdivided into

two identical halves* related by a dyad axis. Note that this argument is based entirely on considerations of symmetry: there is no reason crystallographically why the two parts of the molecule should not be joined by many strong bonds, so that without further chemical information it would be misleading to call them sub-units. However it turns out, in this and some other cases, that there is a correlation between the X-ray and physico-chemical results. Thus horse haemoglobin is known to dissociate into half-molecules in urea solution or on extreme dilution; adult sheep haemoglobin does not so dissociate, nor does it exhibit intramolecular symmetry like that of horse; foetal sheep haemoglobin is probably made up of four identical sub-units, from both crystallographic and physico-chemical evidence. It should be noted that intramolecular symmetry elements need not *necessarily* be revealed in the crystal symmetry: e.g. horse haemoglobin also crystallizes in an orthorhombic form in which the asymmetric unit corresponds to a whole molecule. Another interesting example of the principle is insulin, whose molecular weight is 48,000 in solution at neutral *p*H, but which dissociates at low *p*H's to sub-units of molecular weight 12,000;[5, 6] in the rhombohedral crystal form the unit cell contains three asymmetric units each corresponding to 12,000, while the orthorhombic unit cell is made up of four 12,000 units. It has been proposed by some[7, 8] that the true basic unit is actually 6000, but this has been disputed by others[5, 6]—however no evidence of this smaller sub-unit can be found in the X-ray results: but since negative evidence proves nothing, crystallographers must be neutral in this particular controversy.

As to the cell dimensions themselves, it may be worth repeating the statement often previously made, that little can be *directly* deduced about the shape of the molecule from a knowledge of them, although a more sophisticated analysis of cell dimensions (see p. 269) may under particularly favourable circumstances give some information on this score. In another field, CROWFOOT and DUNITZ[9] have given an excellent example of the dangers of directly associating molecular dimensions and cell size: the molecule of calciferol iodonitrobenzoate, about 25 Å by 5 Å by 7 Å, extends across two unit cells (of dimensions $15 \times 31 \times 7$ Å), and in no way resembles their shape (Fig. 1). In any case, there is an element of arbitrariness in the cell dimensions, which can in theory be chosen at will from an infinite set.

We shall accordingly be concerned only with those proteins whose analysis has been taken further than is indicated in columns 2, 3 and 4 of the Table. The next step has usually been the preparation of Patter-

* The identity is not necessarily complete. Since the diffraction pattern extends only to spacings of 2–3 Å, finer details must escape notice. It would be more correct to say that the two parts are identical if viewed with a resolution no finer than 2–3 Å.

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son syntheses (see below); cases where this has been done are indicated in column 6. From these we may exclude a few which have not been recently or intensively studied; and we are left with a list of proteins, so short that separate tabulation is unnecessary, which may be held to have contributed appreciably to our knowledge of structure. These few, with which the rest of this article will be almost exclusively

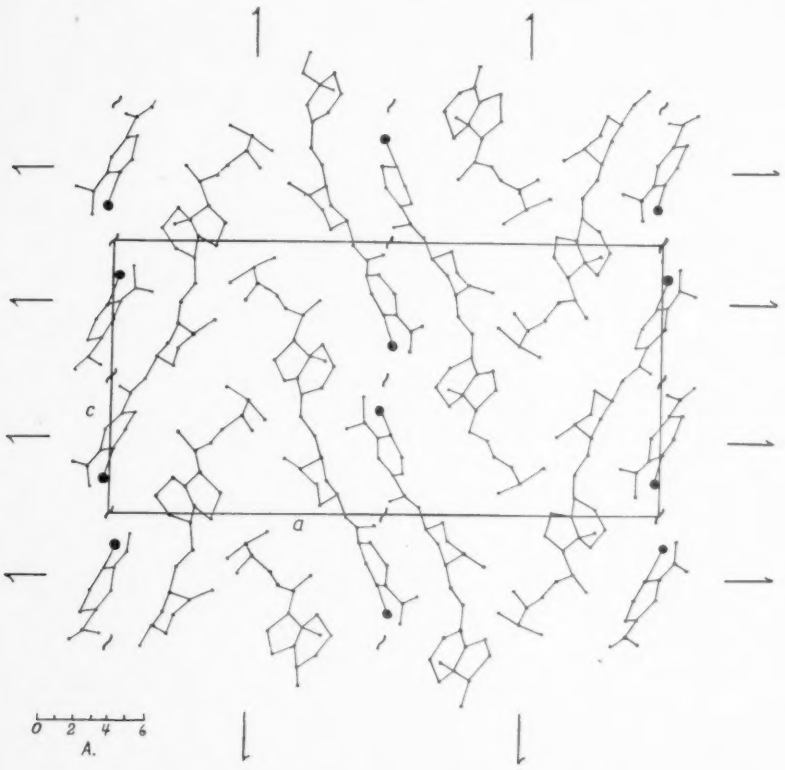


Fig. 1. The crystal structure of calciferol-4-iodo-5-nitrobenzoate, seen in b projection. The unit cell contains four molecules (after CROWFOOT and DUNITZ[9])

concerned, are haemoglobin (PERUTZ, BRAGG and co-workers), insulin (CROWFOOT HODGKIN, LOW and co-workers), β-lactoglobulin (RILEY, CROWFOOT HODGKIN), lysozyme (COREY, DONOHUE, PALMER and co-workers), myoglobin (KENDREW and co-workers), and ribonuclease (CARLISLE, BERNAL and co-workers). It will be seen from the Table that in nearly all these cases more than one form of crystal, often from different animal species, has been studied.

The Patterson (or vector) synthesis

If the unit cell of a crystal contains n atoms we may define n^2 interatomic vectors between all possible pairs of atoms (including n self-vectors of zero length); by transferring all these vectors to a common origin we obtain what is called the vector or Patterson synthesis, which will

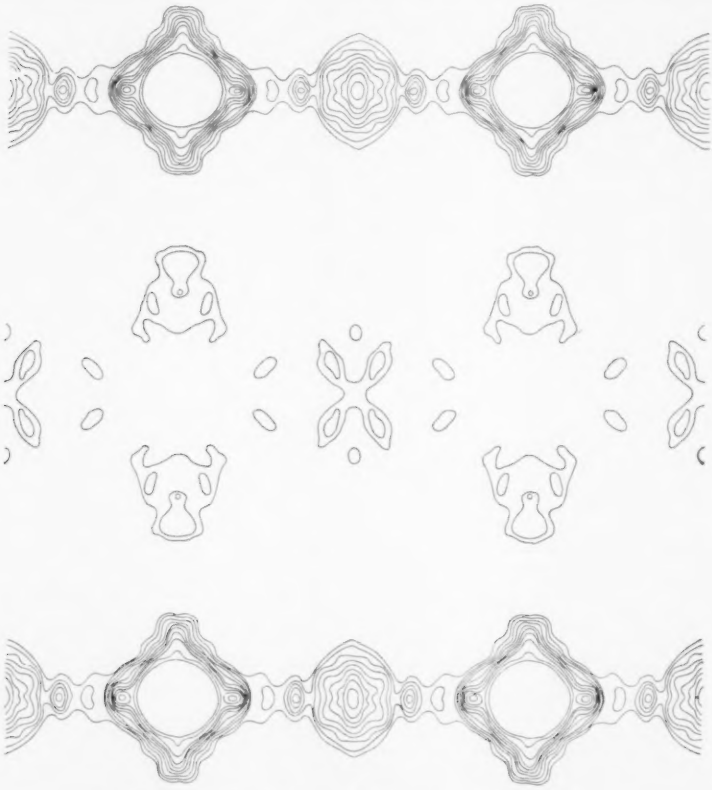


Fig. 2. Examples of layering in Patterson syntheses.
(*a*) Patterson section $P(Oyz)$ of air-dry β-lactoglobulin. $b = 63$ Å approx. (horizontal); $c = 110$ Å approx. (vertical). Origins at $y = 1/4, 3/4$; $z = 0, 1$. Higher contours around origins omitted (after RILEY[18]).

have the same size of unit cell as the original crystal, although its symmetry may be higher (a pair of atoms A and B will always produce two vectors, AB and BA, so that the vector synthesis must have a centre of symmetry at the origin even if the crystal does not do so). It turns out that this synthesis may be derived from the observed diffraction pattern of a crystal without any kind of assumption being made; in this respect it differs from almost any other possible manipulation of the data, and accordingly it has become the classical tool in the x-ray

analysis of protein crystals. And this in spite of the fact that the information with certainty derivable from the vector synthesis is, except in the most favourable cases, of a very limited kind.

Obviously in a complex structure where n is large the n^2 vectors of the Patterson synthesis may produce a pattern so complicated as to be uninterpretable; but if the structure exhibits certain regular features these may be apparent in the Patterson synthesis. Thus a set of

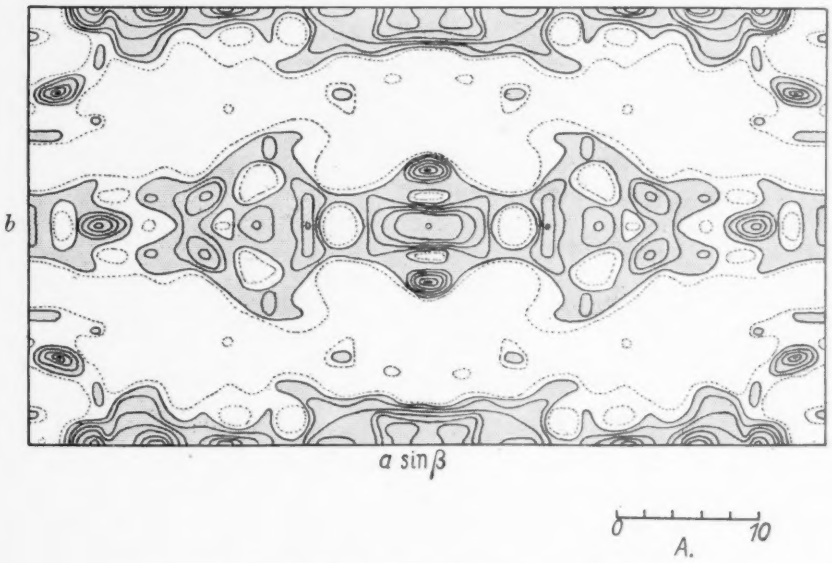


Fig. 2. Examples of layering in Patterson syntheses.
(*b*) *c* projection of horse myoglobin (lattice B). Origin at centre.

equidistant parallel rods (or layers) of high electron density in the crystal will lead to a corresponding set of equidistant parallel rods (or layers) of high vector density, in the same orientation, in the Patterson synthesis. These applications to crystal structures of large molecules, and the type of information they may yield, have been discussed in a recent article,[10] to which reference may be made for simple derivations of the relations involved. In the protein field, unlike some others, these applications are of the most simple and naïve kind; here it will only be necessary to list them, together with recent examples of the employment of each.

It should first of all be remarked that the computation of Patterson syntheses in three dimensions requires a knowledge of the intensities of all the reflexions in the pattern—generally tens of thousands in number—and involves much tedious work which may, however, be greatly reduced by the use of punched-card[11] or electronic[12] calculators:

three-dimensional syntheses have so far been computed only for rhombohedral[13] and orthorhombic[14] insulin, for horse haemoglobin,[15] for lysozyme,[16] for myoglobin,[73] and for ribonuclease.[17] Two-dimensional projections, along a given crystal axis, are much less troublesome; by using a suitable X-ray camera (e.g. the Buerger or Weissenberg cameras) all the data needed for the projection may be contained in a single photograph, and the computation, involving merely a few hundred reflexions, is relatively simple even by hand methods. But of course the information given by projections is much less, and one is more at the mercy of complicated types of packing which may, in unfavourable projections, make the vector structure quite uninterpretable, in the present state of knowledge.

(*a*) *If the contents of the unit cell are arranged in layers of alternately high and low electron density, the Patterson synthesis will also exhibit layers, whose separation and orientation are the same as those in the original structure.* An impressive example of this principle, and one which has been checked by independent evidence, is the (O, y, z) Patterson section of β-lactoglobulin (Fig. 2*a*).[18] The orthorhombic unit cell contains eight molecules; the diagram illustrated is a yz section through the origin of the three-dimensional Patterson synthesis of the dried crystal. The most obvious feature of it is a band of high vector density parallel to b at $z = O$ (i.e. horizontal): between the bands the vector density is low. It is concluded that the molecules lie in rows parallel to b, rows being separated by the whole c dimension (110 Å approx.). This conclusion is strikingly confirmed by DAWSON's electron micrographs of the (100) face of the dried crystal:[19] these show striations parallel to b and 105 ± 5 Å apart, which are evidently the rows of molecules revealed in the Patterson section.

Another example of layering in a Patterson synthesis is provided by monoclinic horse myoglobin,[20] whose a and c projections (Fig. 2*b*) contain regions of alternately high and low projected electron density $b/2$ or 15·4 Å apart. The conclusion drawn is that the crystal structure exhibits similar layering, though of course it does not follow that each molecule is only 15·4 Å thick: there are other possible structures which can give the same appearance in projection.

(*b*) *If, in a given projection, the molecules are so arranged that they overlap very little, it may be possible to detect the main intermolecular vectors.* This is generally not so simple as might at first appear. The molecules are extended objects and are immersed in a medium (i.e. salt solution) whose electron density may differ slightly or not at all from their own mean density; also their intramolecular vector structure contributes greatly to the pattern. In only very few instances can the main intermolecular vectors be identified with any degree of plausibility. It is often the case,

even, that two molecules related by a screw dyad axis—a very common circumstance—may exhibit a negative vector density at the actual point in the Patterson synthesis where the intermolecular vector lies, although the mean density in the region round this point will be above average. One example of a rather plausible identification is shown in Fig. 3, which is the *b* projection of whale myoglobin;[21] round the origin is a high region representing the self-Pattersons of the molecules, while in the cell corners are other high regions having a general resemblance to the origin region. (If the molecules in the cell contain atoms denoted respectively by $a, b, \ldots m, n, \ldots$ etc.; $a', b', \ldots m', n', \ldots$ etc.;

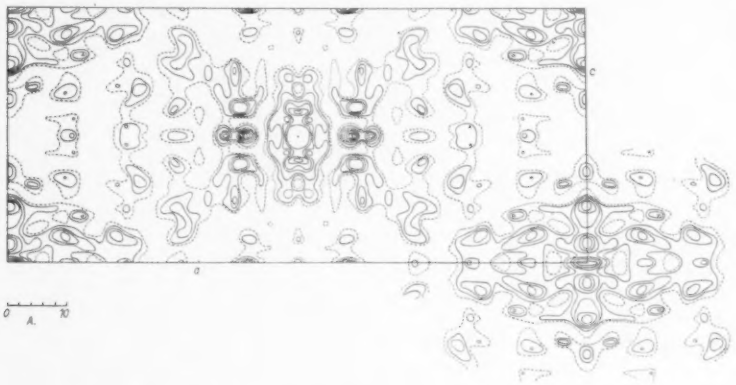


Fig. 3. Example of intermolecular vectors in Patterson syntheses. *b* projection of finback whale myoglobin; origin at centre.

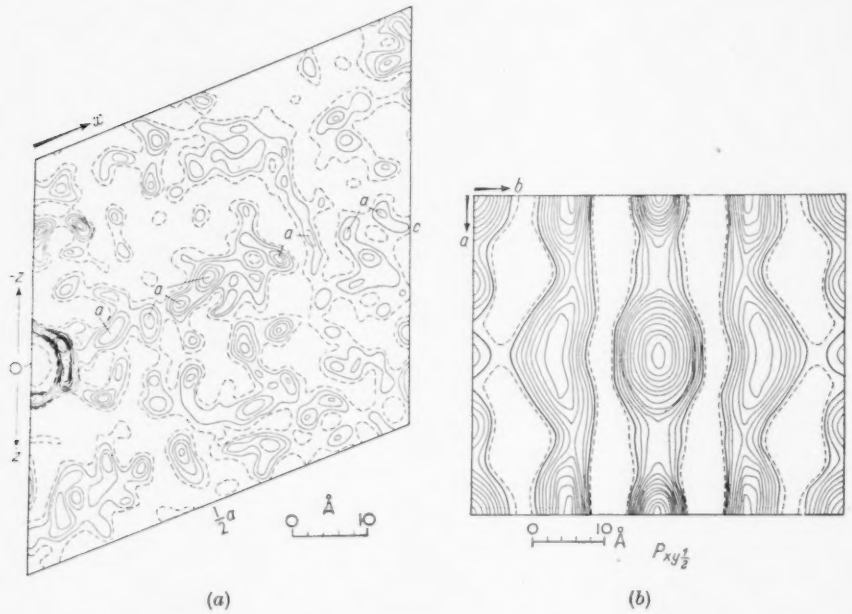
a'', b'', . . . etc. etc., the self-Pattersons of the molecules are sets of vectors such as $\{mn\}$, $\{m'n'\}$, etc.; the cross-Pattersons are sets such as $\{mn'\}$, $\{m''n\}$, etc.) The latter are thought to be the cross-Pattersons; the conclusion is that we have here a pseudo-face-centring, and that in this projection the molecules are related by a vector equal to the semi-diagonal of the cell.

(*c*) *If a set of parallel polypeptide chains constitutes a principal feature of the molecule, and if the molecules are so arranged in the cell that the sets in the different molecules are parallel, then the vector synthesis will also contain indications of rods, parallel to those in the real crystal and the same distance apart.* Rod-like structures in the vector diagram, separated by such a distance that the presumption is that they are derived from sets of equidistant parallel polypeptide chains, have now been reported in at least three prote ns.

1. In *horse haemoglobin*[15] the three-dimensional synthesis (but not the projections) showed rods parallel to *a* and about 10 Å apart, more or less in hexagonal close-packing (Fig. 4*a*).

2. In *orthorhombic insulin*[14] the three-dimensional synthesis showed rods parallel to a and again about 10 Å apart (Fig. 4*b*).

3. In two species of *myoglobin*[20], [21] these rods were observed in projection; in horse myoglobin, in the b projection at 20° to the a axis



(*a*) (*b*)

Fig. 4. Examples of rod-like structures in Patterson syntheses.
(*a*) Patterson section $P(xOz)$ of horse haemoglobin. Origin at O, rods parallel to x through origin (marked a) and 10 Å away. (*b*) Patterson section $P(xy\frac{1}{2})$ of air-dry insulin sulphate; origin at centre. Rods parallel to a (after Low[14]).

(Fig. 4*c*); and in finback whale myoglobin, running parallel to c and very prominently visible (Fig. 4*d*). In each case the rods are about 10 Å apart.

From time to time doubt has been expressed as to the significance, or even the existence, of vector rods in one or other of the above structures. Were only one case known where such rods were reported this scepticism would seem reasonable enough: but the accumulation of instances, albeit of varying degrees of convincingness, leaves little room for doubt, at least in the mind of the author, of their reality. As to whether they really are the vector equivalents of polypeptide chains, one can only say that no one has suggested any other features of the structure to which they might correspond, and that their separation (about 10 Å) and the existence (in well-resolved projections) of antinodes along their length, separated by about 5 Å, suggest a correspondence with the chains of α-keratin, which exhibit similar periodicities.

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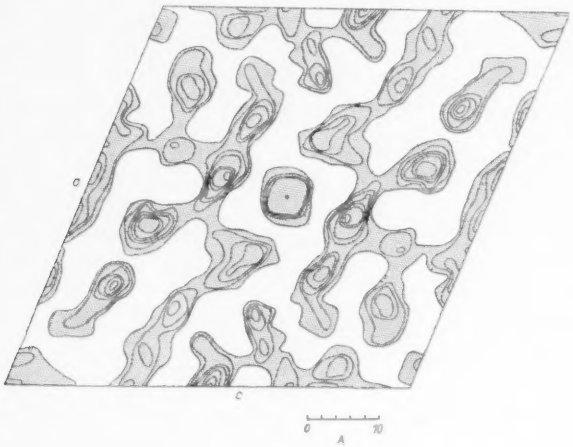
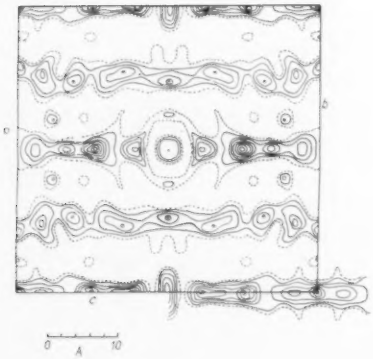
The existence in certain globular proteins (haemoglobin, insulin) of chains similar to those of α-keratin has recently been strongly suggested by studies of their infra-red spectra.[22] Nevertheless the reality or



(*c*)



(*d*)

Fig. 4. Examples of rod-like structures in Patterson syntheses.
(*c*) *b* projection of horse myoglobin (lattice A); origin at centre. Rods 20° to *a*.
(*d*) *a* projection of finback whale myoglobin; origin at centre. Rods parallel to *c*.

otherwise of the rods is so important a question that some kind of quantitative discussion is highly desirable. To carry out such a discussion involves data which have not been generally available for the

following reason. The vector density at a point (x, y, z) in the Patterson synthesis is given by

$$1/V \cdot \sum_h \sum_k \sum_l F^2(hkl) \cdot \cos 2\pi(hx/a + ky/b + lz/c)$$

where the summations are taken over all values of h, k, and l, both positive and negative—and including a term $F^2(OOO)$, the intensity of the radiation scattered at zero angle, i.e. in the direction of the incident x-ray beam. To evaluate this quantity involves measuring the intensities of the reflexions relative to that of the incident beam; ordinary photographic techniques merely give the intensities on an arbitrary scale relative to one another. Comparison with the direct beam may be achieved directly (by Geiger or proportional counter techniques) or indirectly (by comparison of the reflexions with those from a standard crystal of known diffracting power, recorded on the same plate). The term $F^2(OOO)$ is generally omitted from the synthesis; since it is associated with the non-periodic coefficient $cos\ O^\circ$, its omission implies the omission of a certain uniform density of vectors over the whole synthesis—thus it affects the level of the synthesis but not its features.

Direct measurements of absolute intensities have so far, unfortunately, been published only for haemoglobin.[23], [4] With their aid it has been possible to calculate quantitatively[23], [25] how high the vector rods are, on an absolute scale of (electrons/cm^3)^2; and to compare them with what might be expected from any particular molecular model. Of course expectation varies according to the model of the polypeptide chain itself which is assumed to be present; but fortunately the variation is slight—all the models hitherto proposed for α-keratin and the globular proteins have roughly the same electron density in the main-chain regions; the value turns out to be a good deal higher than the average for the molecule as a whole. The reason for this is that in the main-chain nearly all the atoms are separated by covalent bonds, whereas in the peripheral side-chain regions, although the bonds in each side-chain are covalent, each side-chain is separated from its neighbours by Van der Waals distances, which are considerably greater; hence the mean electron density (a function of the cube of the interatomic distances) of the main-chain regions is much higher than that of the side-chain regions.

It turns out that the vector rods are definitely much less high than would be expected if the molecule of haemoglobin consisted entirely or almost entirely of parallel lengths of chain. To resolve the discrepancy it must be assumed, either that the chains are irregular, or that they are not absolutely parallel, or that rather less than half the molecule consists of chains parallel to the rod direction. The first of these assumptions seems rather improbable, at any rate if the now-favoured α-helix[26] be assumed to be present (as was done in these analyses): the α-helix

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appears to be a rather rigid structure. The other two assumptions appear to be very plausible, however: it is now known that the number of independent open chains in the molecule (six)[27] is much less than the number of rods (about 13–17) required to make up a roughly globular molecule on the simple model. Hence the chains must turn corners; if cyclic polypeptides (not detectable by the end-group method) are present they too must contain "corners"; further, there is evidence (see p. 246) that the haemoglobin molecule is made up of sub-units, probably two or four in number, and the more sub-units there are, the more corners there are likely to be. At this stage of the analysis it is not possible to be more precise; the present state of affairs would seem to be that the molecule cannot possibly consist simply of straight and parallel lengths of chain, either on general grounds or from the X-ray evidence—on the other hand the vector rods are not reduced to insignificance even by ruthless analysis;[25] they really do exist and are significant, so we must conclude that a considerable part of the molecule does consist of more-or-less parallel chains.

Recently CRICK[97, 98] has shown that it is very plausible to suppose that α-helices pack together at angles of 20° to one another, rather than in strictly parallel array; in this way the side-groups of neighbouring chains can be made to interlock with each other like the meshing of gears. Although this hypothesis was proposed with particular reference to fibrous proteins, it is equally applicable to globular proteins if indeed these are built of α-helices, and if confirmed it would provide another reason why the vector rods in Patterson syntheses are less high than might at first be expected.

The measurement of absolute intensities for other proteins is very important, since without them it is generally not possible to discuss the plausibility of any proposed structural model in more than qualitative terms. So far as the author is aware the only protein other than haemoglobin for which direct measurements have yet been made is insulin;[28] these are still unpublished. In the recent study of lysozyme[16] there is a detailed discussion of an indirect way of obtaining absolute intensities by applying a method developed by WILSON,[29] whereby an examination of the statistical distribution of the relative intensities as a function of spacing can give a value of $F^2(OOO)$; a similar method was earlier applied in a less sophisticated form in the haemoglobin work.[15] It is however still by no means clear that this method is capable of accurate results when applied to protein crystals, and in the meantime it would appear that direct determinations are much to be preferred when they are practicable. In lysozyme, in any case, vector rods attributable to polypeptide chains are not observed in the three-dimensional Patterson synthesis; they would in fact not be expected to appear since, in consequence of the rather complicated

crystal symmetry, the eight molecules of the unit cell do not in the general case have the same orientation.

Before leaving the subject of vector equivalents of polypeptide chains we may notice that if a crystal containing parallel chains in approximately hexagonal close-packing is viewed in the projection in which the chains are end-on, we should expect to be able to see in the corresponding Patterson projection hexagonal arrangements of peaks corresponding to inter-chain vectors. Such arrangements have in fact been seen in a number of cases, e.g. in insulin,[14] horse haemoglobin,[15] and finback whale myoglobin[21] (Fig. 5). It would appear, in fact, that the

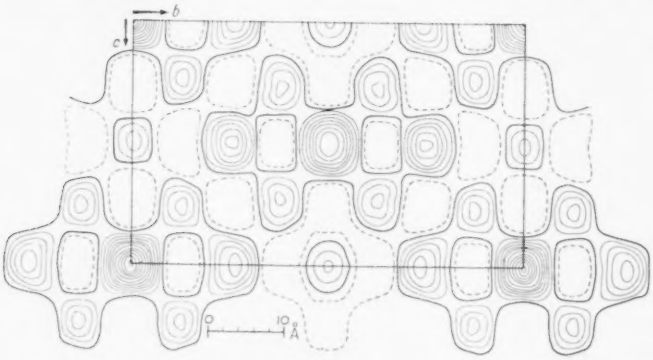
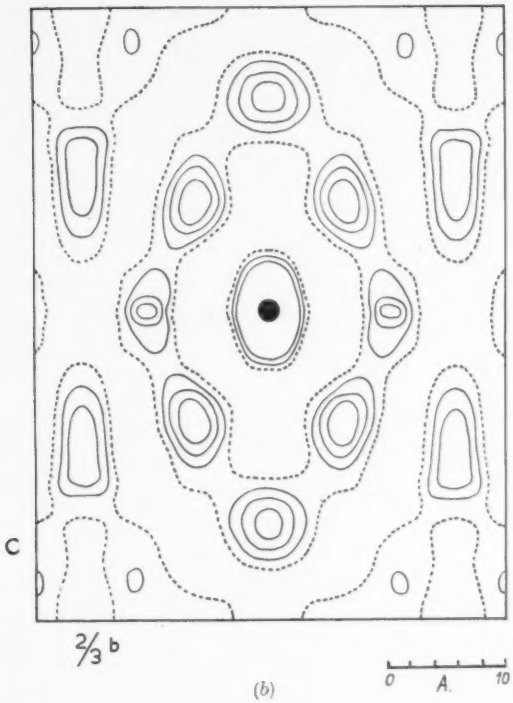


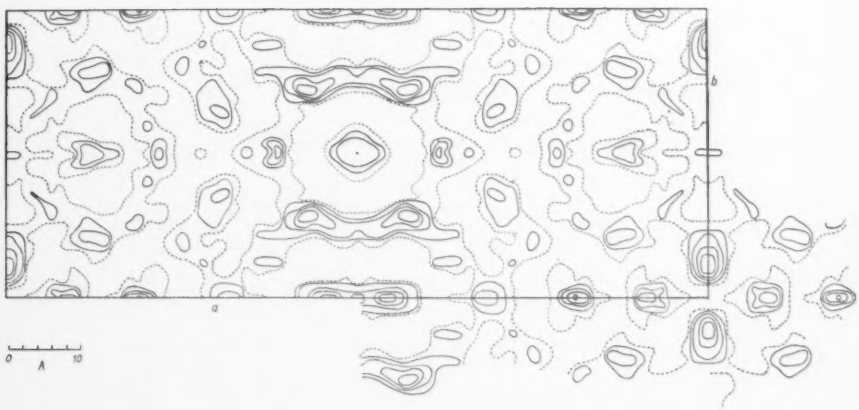
Fig. 5. Examples of end-on projections of vector rods in Patterson syntheses. (*a*) *a* projection of air-dry insulin sulphate; origins at corners (after Low[14]).

existence of such an arrangement in a particular projection may provide *prima facie* evidence for the predominant chain direction when other evidence is lacking; for example the nature of the *c* projection of ribonuclease (Fig. 6*a*) suggested that the predominant chain direction was *c*, even though the three-dimensional vector synthesis very surprisingly does not exhibit prominent rods.[3], [17] Arguments of this kind must be used with discretion, however; the basal projection of rhombohedral insulin shows very well-developed hexagonal arrangements of peaks,[30] suggesting a chain direction parallel to the trigonal axis, but recent infra-red evidence suggests that they are in fact nearly perpendicular to that axis.[22] More than one example has now been found to show that hexagonal arrangements of peaks in projection can be produced from crossed sets of rods nearly perpendicular to the direction of projection.

(*d*) *If two protein molecules are in some respects similar, the vector syntheses of their crystals may also resemble one another, provided the packing arrangements are also similar.* It should by now be clear that we are only able to interpret Patterson syntheses of proteins in very



(b)



(c)

Fig. 5. Examples of end-on projections of vector rods in Patterson syntheses.
(*b*) *a* projection of horse haemoglobin: origin at centre (after PERUTZ[77]).
(*c*) *c* projection of finback whale myoglobin; origin at centre.

favourable cases, and then in the most partial way. However, as more of these syntheses have been computed, family resemblances between certain of them have become apparent; our knowledge of, so to say, the comparative geography of protein vector maps has gradually increased and has enabled us occasionally to classify together related structures

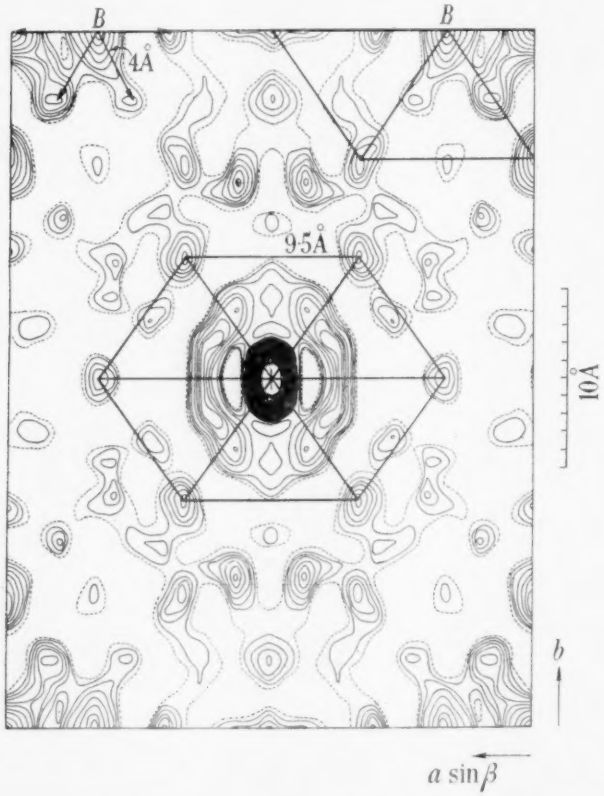


Fig. 6. Further examples of end-on projections of vector rods.
(*a*) *c* projection of ribonuclease (after CARLISLE and SCOULOUDI[33]). Origin at centre.

and related projections, even if we cannot always interpret them. In the last section we have already noted two important types of projection, which have been interpreted as rods viewed sideways and rods end-on. These may be recognized in other proteins less completely analysed; thus human haemoglobin[31] gives an *a* projection (Fig. 6*b*) which, in the central region, is astonishingly similar to the "end-on" projection of horse haemoglobin which has already been discussed (Fig. 5*b*); the *a* projection of ox haemoglobin[32] (Fig. 6*c*) is similar,

in spite of the fact that its unit cell is twice as large as those of the former two.

Again, it has been observed[33] that the three-dimensional syntheses of horse haemoglobin and of ribonuclease are remarkably similar in respect of the regions immediately surrounding the origin: in each

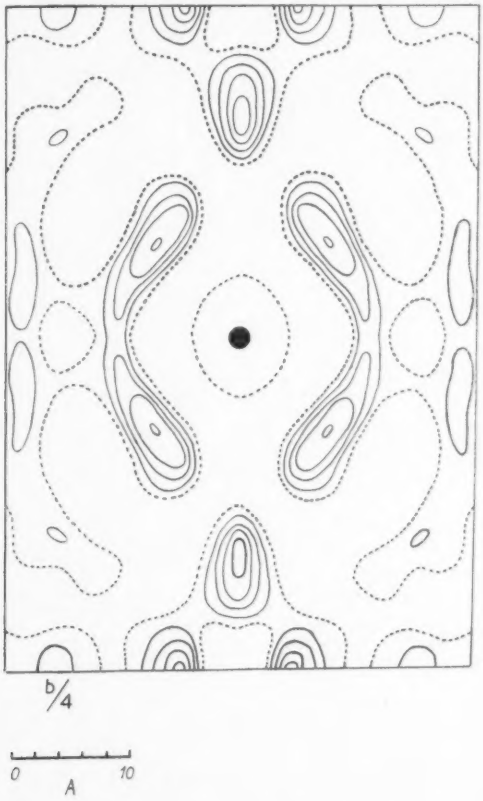


Fig. 6. Further examples of end-on projections of vector rods. (*b*) *a* projection of human haemoglobin (after PERUTZ and TROTTER[31]). Origin at centre.

there is a shell of high vector density at about 5 Å from the origin, and the irregularities in the shell are very similar in the two. It is unfortunate that this shell, which is one of the most prominent features of the pattern, has not received any adequate interpretation in terms of the type of structure so far discussed, although some discussion of it has been given[34] in connexion with a quite different conception of protein structure.

An example of a slightly less direct relationship between vector projections is provided by horse myoglobin. It was recently found[35] that this protein sometimes crystallizes in a pseudo-orthorhombic form whose unit cell is *exactly* twice as large as the normal monoclinic one, this doubling being achieved by making $c \sin \beta$ twice as great, all the

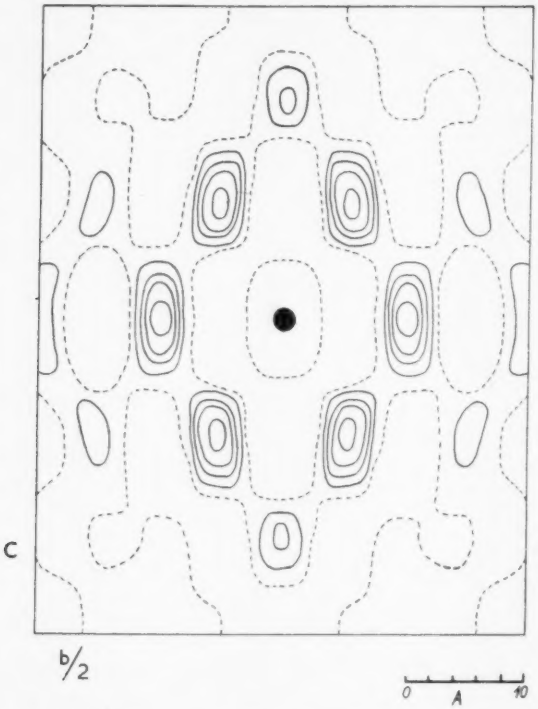


Fig. 6. Further examples of end-on projections of vector rods. (*c*) *a* projection of ox haemoglobin (after CRICK[32]). Origin at centre.

other dimensions remaining constant; it thus contains four molecules per cell instead of two. The *b* projection of the new form (Fig. 7*a*) does not at first appear to resemble that of the monoclinic form (Fig. 4*c*) in the slightest degree. However, the simplicity of the relations between the cell dimensions suggested that the pseudo-orthorhombic form might be derived from the monoclinic as shown in Fig. 7*b*, i.e. by taking alternate rows of unit cells and, by introducing dyad axes parallel to *a*, inverting them. If this is so one expects the origin region in the pseudo-orthorhombic projection to resemble closely a composite projection made by superposing the monoclinic *b* projection and its inverse (about *a*). Fig. 7*c* shows that indeed the resemblance is astonishingly

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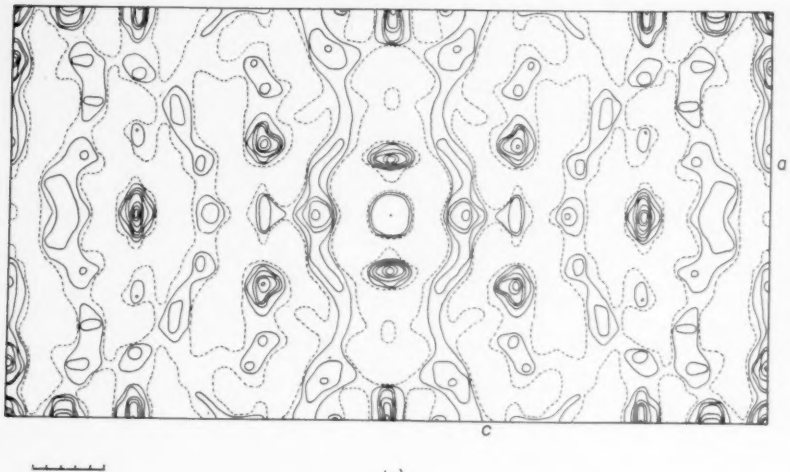
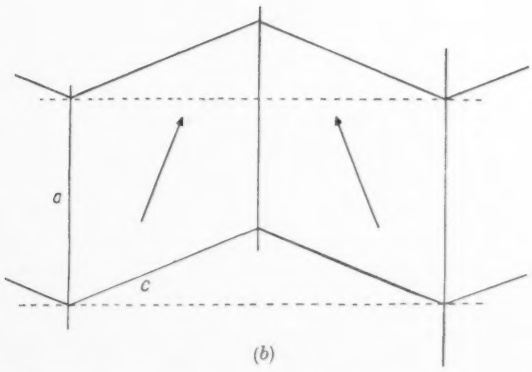
close. The conclusion would be that the unit cell contains two pairs of molecules, those in each pair being related to one another in the same



(*a*)



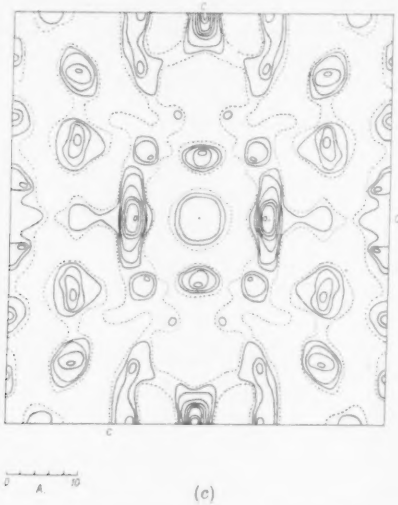
(*b*)

Fig. 7. (*a*) *b* projection of pseudo-orthorhombic horse myoglobin. Origin at centre.
(*b*) Suggested relationship between unit cells of monoclinic and pseudo-orthorhombic horse myoglobin, showing "twinning" of *c* face of monoclinic cell (full lines) to produce pseudo-orthorhombic cell (dotted lines).

way as those in the monoclinic form, i.e. with parallel polypeptide chains (see p. 252), and the pairs being related so that the chain directions cross at an angle twice that between *a* and the chain direction in the monoclinic form, i.e. about 40°. It is interesting to observe that if

the pseudo-orthorhombic form alone were known it is certain that its *b* projection would not have been interpretable in this sense.

To summarize, we are beginning to recognize certain families or types of protein Patterson synthesis, and to appreciate relationships between apparently dissimilar ones: sometimes the meaning of a particular feature is understood, or at least dimly guessed at; in other cases there is as yet no interpretation, nevertheless the possibility of a



(*c*)

Fig. 7. (*c*) Composite *b* projection of monoclinic horse myoglobin, derived by superimposing normal *b* projection (Fig. 4*c*) on its mirror image

classification, which has only become real in the last year or two, marks a significant advance. On the other hand increasing experience has also shown that appearances may be deceptive and that apparent simplicity may conceal real complexity, and *vice versa*.

Evidence relating to the polypeptide chain configuration in globular proteins

The controversy which has gone on for twenty years about the nature of the chain configuration in proteins is still being vigorously waged. In the case of synthetic polypeptides and α-keratin there has during the past year been a growing body of opinion which favours the recently-proposed α-helix of PAULING, COREY and BRANSON:[26] the evidence has been summarized in a recent review.[36] For the globular proteins, with which alone we are concerned here, the evidence is still rather sketchy and not entirely consistent.

(*a*) We have already remarked on the resemblance between the periodicities of the vector rods in globular proteins and those of the α-keratin chain, and also on the correspondence between their infra-red spectra (p. 252); these similarities lend at least presumptive support to the idea that the chain configuration is the same in the two types of protein. The provisional conclusion would be that, if the α-helix be accepted as the basis of the structure of α-keratin, it must also be present in the globular proteins.

(*b*) Strong support for the α-helix was provided by the discovery of a powerful 1·5 Å meridional reflexion in a number of fibrous proteins believed to have the α-keratin structure.[37], [38] A similar reflexion, albeit a very weak one, was observed in haemoglobin.[37] It is surprising that in ribonuclease, whose diffraction pattern extends to unusually large angles (corresponding to spacings of less than 1 Å) and has been thoroughly explored, there is no trace of strong reflexions at 1·5 Å.[39] Now it is true that in a particular case pairs of chains might be arranged so that a meridional 1·5 Å reflexion was cancelled out (e.g. if they were displaced relative to one another by 1·5/2 Å axially); but in such a case near-meridional reflexions of about the same spacing should be observed. The difference between the two proteins is the more remarkable in view of the resemblance between their three-dimensional vector syntheses which we have already noted (p. 259).

(*c*) Further evidence favouring the α-helix has come from studies of the radial distribution function of globular proteins. This is analogous to the Patterson function, but it relates to scalar instead of to vector quantities; whereas the Patterson function gives the distribution of interatomic *vectors* in three-dimensional space, the radial distribution function is the distribution of interatomic *distances*. If the three-dimensional Patterson synthesis is available, the radial distribution function may obviously be calculated from it by integration over a series of spherical surfaces centred on the origin: this calculation has been performed for haemoglobin[40] using the three-dimensional data we have already discussed.[15] Alternatively the function may be derived directly from x-ray data by averaging over the whole three-dimensional pattern of a single crystal or, more directly, by measuring the distribution of scattered intensity from an amorphous specimen as a function of angle. This has been done by Riley and Arndt for serum albumin[41] and other proteins.[96] In each case the distribution agrees well with that calculated[40] for the α-helix, in spite of the fact that the side-chains (except the β-carbon atoms) must perforce be omitted from consideration. This is evidence for the presence of the α-helix in these two proteins. It seems impossible to assess the strength of the evidence, however, until radial distribution curves have been calculated for other proposed chain models; in no other cases are these available, so there is

at present no way of knowing how sensitive is the form of the radial distribution function to the type of configuration postulated.*

(*d*) We have noted the absence of strong 1·5 Å reflexions in the diffraction pattern of ribonuclease. The suggestion has been made[17] that the data for this protein fit better the so-called α_{II} configuration.[43] The process by which this conclusion was reached is as follows: the "end-on" (*c*) Patterson projection (Fig. 6*a*) suggests plausible packing arrangements for the rods; these are used to calculate electron-density maps (see the next section); and in these maps the peaks corresponding to the rods are found to consist of doublets, the components being about 4 Å apart. The end-on view of the α-helix, at low resolution, is simply circular; that of the α_{II} structure is a double peak with components separated by 4 Å as observed.

It seems impossible at the present time to reach definite conclusions about the chain configuration in the globular proteins. On general grounds one might hope that the same configuration would predominate in all of them; and this simple hypothesis is supported by the similarity between the infra-red spectra, and between certain features of the Patterson syntheses, of ribonuclease and haemoglobin. On the other hand, as has been noted in *b* and *d*, there appear to be serious difficulties about interpreting other features of the X-ray data for the two proteins in terms of the same structure.

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The direct interpretation of "end-on" Patterson projections

If it be assumed that the prominent peaks in the projections illustrated in Figs. 5 and 6 are really due to a more or less hexagonal arrangement of chains viewed end on, it should be possible, by regarding the lump of projected electron density corresponding to each chain as an "atom," to apply the classical methods of crystallography in an endeavour to disentangle the Patterson projection; or, in other words, by selecting arrangements of "atoms" which seem plausible (as judged by the inter-chain vectors in the observed Patterson projections) to calculate the phases of the reflexions and hence to work out an electron density projection, and by successive refinements to reach a structure giving good agreement between observed and calculated intensities. In view of the facts that all discussion of the side-chains (amounting to about half the total bulk of the protein) is omitted, and that we know it to be a great over-simplification (at least in haemoglobin; see p. 254 above) to regard the molecule as consisting entirely of parallel sets of chains, it is

* I have recently been informed by Dr. D. P. RILEY that he has now calculated the radial distribution curve for the chain configuration described by BRAGG, KENDREW and PERUTZ[42] as 4_{13}, and that it is materially different from the observed curve. Similar calculations have recently been carried out by Dr. JERRY DONOHUE for other chain models (private communication). At the time of writing it is, however, still too early to assess the weight of the evidence provided by such curves.

not to be expected that perfect agreement will be obtained, and the results of such analyses must be regarded with considerable scepticism until they are supported by other evidence.

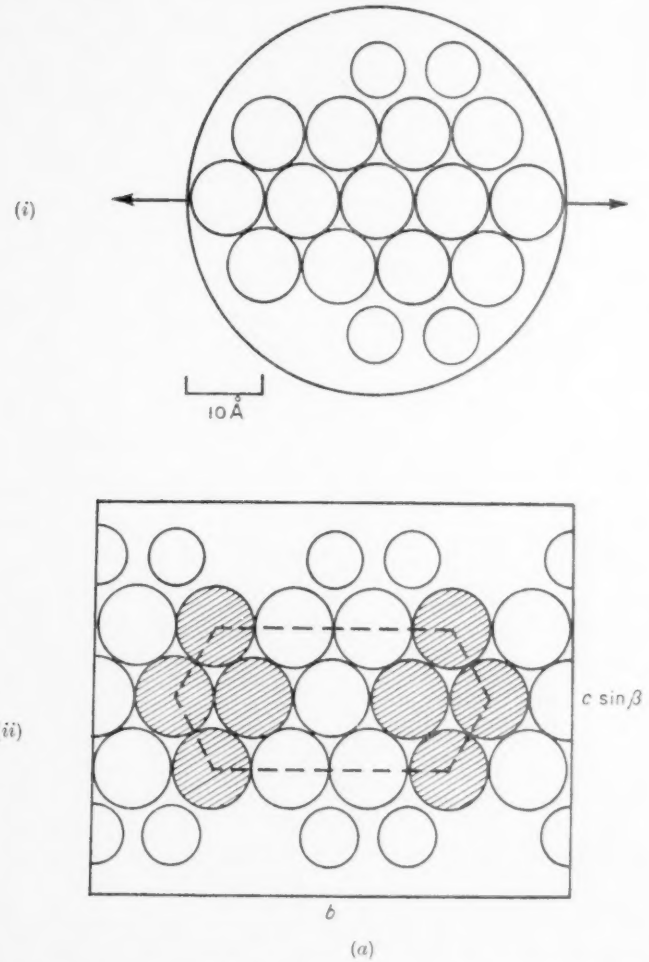
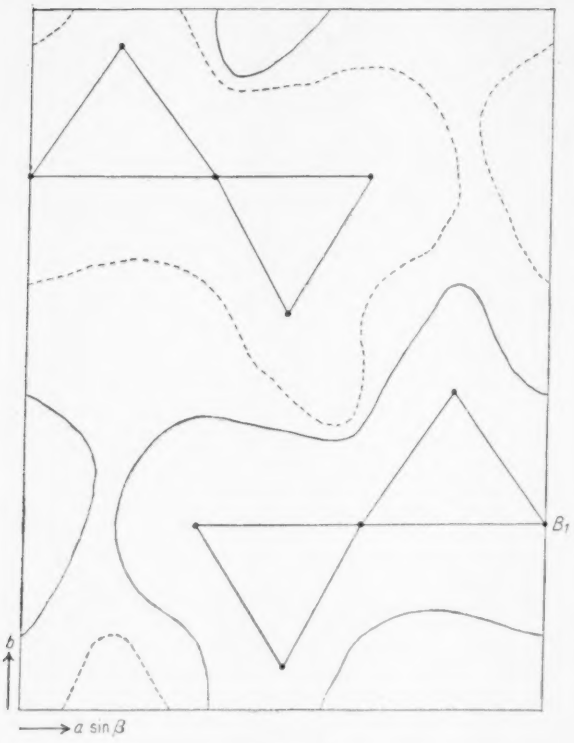


Fig. 8. Tentative models of protein molecules: end-on views. (*a*) Horse haemoglobin; (*i*) arrangement of chains in single molecule, (*ii*) view of unit cell—overlapping chains are shaded. (After Bragg, Howells and Perutz[22]).

The most elaborate analysis of this kind so far attempted is that of haemoglobin,[23] the tedious computations involved here being greatly shortened by the use of the "fly's eye" and the XRAC analogue computer. The model arrived at is shown in Fig. 8*a*; it differs from that earlier proposed[15] in that it contains *five* layers of chains instead of



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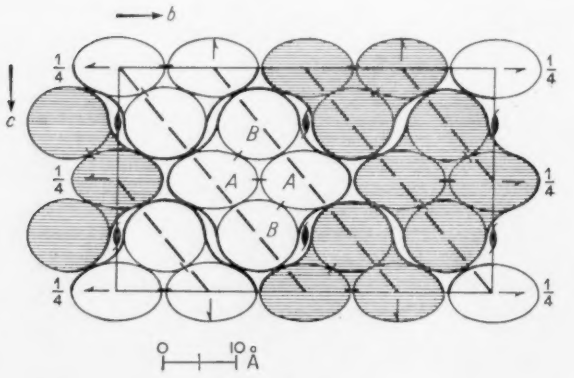

(c)

Fig. 8. Tentative models of protein molecules: end-on views. (*b*) Ribonuclease. The unit cell contains two molecules, each consisting of five chains (after CARLISLE and SCOULOUDI[3]). (*c*) Insulin (after LOW;[44] this is one of two similar models which have been tentatively proposed).

four. A similar example, to which reference has already been made (p. 264), is the analysis of ribonuclease,[3], [17] which leads to the model shown in Fig. 8*b*. The insulin "end-on" projection has also been analysed in this way,[44] giving the model of Fig. 8*c*.

The external shape of the molecule: changes in salt concentration[24]

Haemoglobin crystals are generally prepared in strong salt solution, but it is also possible to make them from salt-free media, with the same cell-dimensions as the salt-containing ones. Much evidence has accumulated[45], [46], [47] that the external salt is in dynamic equilibrium with that inside the crystal; provided the *protein* concentration in the external medium is high enough to prevent the crystal dissolving, the salt content of the crystal can be altered at will by altering the salt concentration outside (within certain limits, beyond which the crystal often changes discontinuously to a different lattice with new cell dimensions). If such variations are made, it is found that the intensities of the low-order reflexions of the diffraction pattern also change, but not those of the high orders. The former are due mainly to the *inter*molecular, and the latter mainly to the *intra*molecular structure of the crystal; and on this as well as on other grounds it has been concluded that the salt does not penetrate the molecules themselves, but remains in the inter-molecular liquid regions. If now salt is gradually introduced into a crystal containing water (electron density 0·33 el/Å^3), the electron density of the liquid region rises towards that of the protein (mean value 0·405 el/Å^3 for protein plus hydration layer) and the contrast between the protein and the liquid region decreases: accordingly the intensities of the low-angle reflexions also in general decrease, proportionally to the increase in the electron density of the liquid. Now since a uniform distribution of electron density throughout the cell has no effect on any of the reflexions (except *OOO*), an increase of the density everywhere outside the molecule has the same effect on the reflexions as decreasing it by an equivalent amount inside them. Hence an amplitude $|F| = |F(water)| - |F(salt)|$ may be taken to correspond to an assembly of "molecules" of *uniform* electron density ($\rho_{salt} - \rho_{water}$) suspended in a vacuum. (At this stage in the argument the assumption is made that the molecule is centro-symmetrical.) These "molecules" have the same shape as the real ones but have no internal structure: hence by this method the diffraction effects of the shape can be discussed independently of the internal structure.

We now digress briefly to discuss the diffraction from a crystal in terms of Fourier transforms. The Fourier transform of any object may be thought of as derived by analysing the object into its sinusoidal Fourier components (by the standard procedures of Fourier analysis),

and then arranging them in an array, in order of decreasing wavelengths. In optics the transform may be directly observed: it follows from standard theory that the diffraction pattern of an object is in fact its Fourier transform, the least deflected rays corresponding to the components of longest wavelength, and the most deflected to those of shortest wavelength. The simplest case is that of a slit, where the transform consists of a large central maximum, and on either side of it a set of smaller maxima and minima. Now if the diffracting object is many times repeated in a regular manner, the interferences between successive elements cause a set of supplementary maxima and minima (whose spacing is reciprocal to the distance between the elements) to be superimposed on the transform, breaking up the diffraction pattern into a set of fringes. The classical example is the diffraction grating, which may be thought of as a series of equidistant slits; the successive orders of diffraction are the fringes, and their progressive changes in intensity correspond to the change in the value of the Fourier transform.

In a crystal the argument must be generalized to three dimensions. The Fourier transform of a single molecule is just its x-ray diffraction pattern: a repeating pattern of molecules—i.e. a crystal—produces a diffraction pattern consisting of the transform crossed by sets of sharp maxima with spacings reciprocal to the repeat distances of the lattice. These maxima are in three dimensions—in other words a set of points in a three-dimensional array (known as the reciprocal lattice). In two dimensions these relations may be illustrated conveniently by means of the Fly's Eye, an optical analogue of the x-ray diffraction camera. Here the crystal (or rather a two-dimensional projection of it) is represented by sets of holes in a black card (Fig. 9, insets); the diffraction pattern of a single array of four holes—i.e. the Fourier transform of a single "molecule"—is shown in Fig. 9*a*; if two "unit cells" are used this transform is crossed by a set of fringes (Fig. 9*b*), while four "unit cells" arranged rectangularly give two sets of fringes at right angles (Fig. 9*c*). With a large number of unit cells the fringes narrow up to give sharp "reflexions" whose spacing corresponds to the reciprocal lattice (Fig. 9*d*); if the arrays are placed closer together the reciprocal lattice expands, a different set of points in the transform being picked out (Fig. 9*e*).

Finally it must be observed that in a crystal the repeating unit (or unit cell) may be—indeed almost always is—made up of a number of molecules in different orientations. The example illustrated in Fig. 9 is a particularly simple one, since in the projection shown the "unit cell" is just a single molecule, so that the transform of the unit cell is the same as that of the molecule alone. In general this is not so, and it is necessary to compound the transforms of individual molecules in the correct orientations and phase relationships to obtain the transform of

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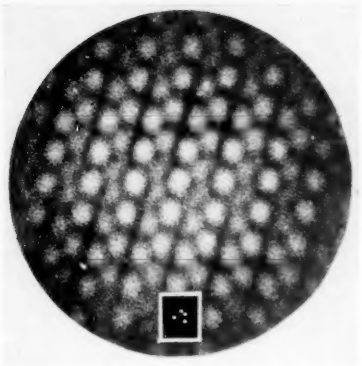
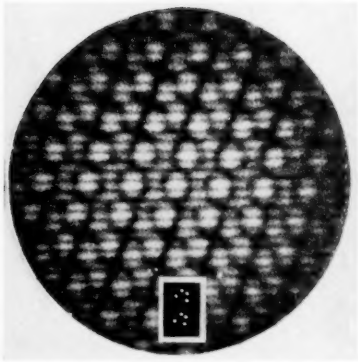


(*a*)



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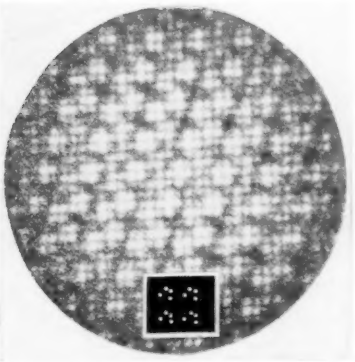

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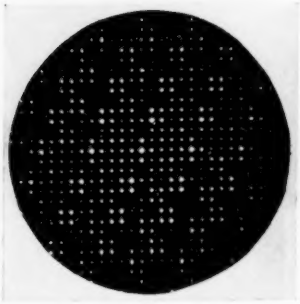

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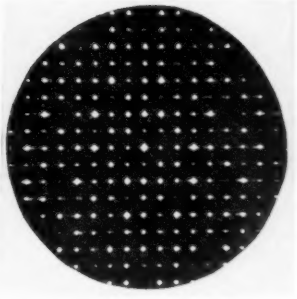

(*e*)

Fig. 9. (*a*) Optical diffraction pattern of the "molecule" shown in the inset. (*b*) Diffraction pattern of two "molecules" arranged as shown in inset. (*c*) Diffraction pattern of four "molecules" arranged as shown in inset. (*d*) Diffraction pattern of a large number of "molecules," in the same relation to one another as those in the inset to (*c*). (*e*) As (*d*), but with molecules nearer one another. (After LIPSON and TAYLOR[48].)

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the whole cell. As we shall see later, the problem of solving the structure of complex crystals may be much simplified in cases where all the molecules in the unit cell have the same orientation and known relative positions, so that the transform of the whole cell may immediately be derived from that of the molecule.

To return to the hypothetical structureless haemoglobin "molecules," the diffraction pattern of a crystal built up of these objects is arrived at by sampling the values of the Fourier transform of a single "molecule" at the points of the reciprocal lattice. To a first approximation we may regard the "molecules" as ellipsoids: the Fourier transform of an ellipsoid consists of a large central maximum, ellipsoidal in shape (its axes inverse to those of the real ellipsoid) surrounded by a considerable negative region, which is surrounded in turn by smaller fringes. By working only in the central region it is possible to find out roughly where the Fourier transform of the "molecule" changes over from first maximum (positive) to first minimum (negative), by noting that at this point the F values must be small, while inside it they will be large (and positive) and just outside it they will also be large (and negative). It can be shown that, provided consideration is given only to the regions of first maximum and first minimum, the argument would still be valid if the actual shape departed from that of an ellipsoid to the extent of being, for example, a parallelopiped or a cylinder.

In this way it was possible to outline the contours of the central maximum of the Fourier transform, and hence by the application of ordinary diffraction theory directly to calculate the dimensions of an ellipsoid equivalent to the "molecule." It turned out that in haemoglobin the dimensions of the ellipsoid were 55 Å in each of the b and c directions and 65 Å in the a (or chain) direction. It should be noted that this ellipsoidal "molecule" is equivalent to the real molecule plus any hydration layer into which salt does not penetrate.

The external shape of the molecule: packing arguments

We have remarked on the dangers attendant on any naïve attempt to deduce molecular shape from cell dimensions. Haemoglobin is, however, a uniquely favourable subject for such an attempt,[49] since measurements have now been made of the cell dimensions of at least seventeen different forms of the protein, derived from several different species (most of which exhibit polymorphism), and some of them available in several different shrinkage stages. Two assumptions have to be made as a preliminary. The first is that haemoglobins from different species closely resemble one another, at least as regards external shape: this is plausible on general grounds and is confirmed by the almost complete self-consistency of the argument which follows. The second is that in all crystal forms and at all states of shrinkage the molecules make a

maximal number of contacts in a close-packed arrangement. The latter assumption is radically different from that made in the earlier work on haemoglobin[47], [50] which suggested that the structure consisted of layers of protein molecules completely separated by layers of water. This assumption was first questioned by DORNBERGER-SCHIFF,[51] who showed that the earlier data could be equally well explained on the basis that the molecules were close-packed at all stages of shrinkage, sliding past one another in the transition from one stage to the next.

Briefly the method is to take into account any evidence available as to the orientation of the molecules in any particular form (e.g. from a

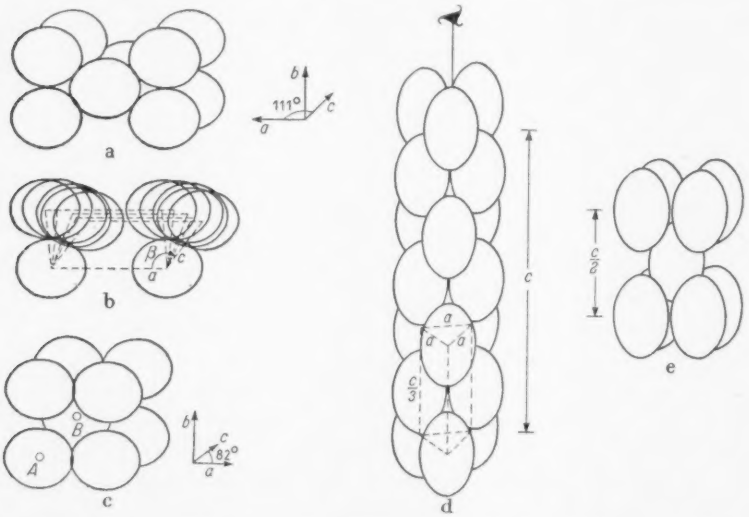

Fig. 10. The packing of haemoglobin molecules into various unit cells. (*a*) Monoclinic horse haemoglobin—normal wet stage. (*b*) Projection of successive shrinkage stages of the above on the *b* face, showing molecules sliding past one another. (*c*) Reduced human haemoglobin (monoclinic). (*d*) Reduced horse haemoglobin (hexagonal). (*e*) Human oxyhaemoglobin (tetragonal). (In (*d*) and (*e*) the dimensions of the ellipsoids are slightly different from those discussed in the text, and are those proposed at an earlier stage of the research. The most recent values do not alter the general nature of the packing. After BRAGG and PERUTZ[49])

study of the optical properties which may reveal the orientation of the haem groups, and from the crystal symmetry elements which may impose restrictions on the packing), and then, assuming close-packing, to try to find a shape of ellipsoid which will pack equally well into *all* the forms studied. It turns out that an ellipsoid of the shape deduced by the method discussed in the last section will in fact pack into all the unit cells examined (with one exception, namely foetal sheep

haemoglobin), the optical and symmetry conditions being satisfied in each case. It is difficult to be certain that this solution is a unique one, but the range of forms examined is such that it appears at the least to be very probable. In one instance (reduced horse haemoglobin, salt-free) the mode of packing proposed is strikingly confirmed by analysis of the intensities of the low-order reflexions. Some of the packing arrangements proposed are illustrated in Fig. 10.

A slightly different and very interesting discussion of the connexion between packing and molecular shape has been given in connexion with the analysis of lysozyme,[16] though it has not yet yielded results sufficiently precise to enable a definite model to be proposed with confidence. The method is to place spheres, each corresponding to one molecule, in general positions in the unit cell, the parameters being so chosen that the spheres are close-packed; from the centres of these spheres an idealized point-Patterson of inter-molecular vectors is constructed. The spheres are then shifted slightly in such a way that the points of the idealized Patterson correspond with regions of high vector density in the real Patterson synthesis. In this way some, but not all, of the regions of high density in the Patterson synthesis were accounted for; a more complete agreement was obtained by adding a second set of scattering centres in new general positions near the first, the two together making a kind of doublet or elongated molecule. In parallel, calculations were made of the intensities of a few of the most striking low-order reflexions, from models of the unit cell built up of spherical and ellipsoidal molecules. In the end good agreements were obtained, both between idealized and real Patterson syntheses, and between calculated and observed intensities, with ellipsoidal molecules of dimensions $38 \times 24 \times 24$ Å. The agreements are very encouraging; nevertheless the authors do not make more than tentative claims for this model.

Phase determination by the correlation of shrinkage stages[52]

The method now to be described, which has recently been applied to haemoglobin, differs from the two previous ones in that it is capable of giving information about the *internal* structure of the molecules. Its application depends on a number of fortunate properties of monoclinic horse haemoglobin crystals. One is that they shrink (in a series of discrete steps) when dehydrated; another, that the symmetry conditions of their particular space-group ($C2$) require that the two molecules in the unit cell have identical orientations relative to the axis; in fact this relative orientation remains almost the same at all stages of shrinkage.

It has been pointed out (p. 267) that the diffraction pattern may be derived by sampling the Fourier transform of the unit cell at points

corresponding to the reciprocal lattice points; this could be done for any shrinkage stage by using the correct reciprocal lattice for each. Since in this case the two molecules have the same orientation, and are in special (i.e. known relative) positions, the transform of the whole unit cell can immediately be derived from that of a single molecule (provided due allowance is made for the uniform liquid regions in the unit cell; this can be done without difficulty). Hence the diffraction pattern of the whole crystal could be derived from the transform of a single molecule by sampling with the reciprocal net appropriate to the shrinkage stage under consideration. Conversely, if the amplitudes of all the reflexions in all the stages are plotted on the same diagram in reciprocal space, the result is a map of the Fourier transform of a single molecule;* it would become more complete the more shrinkage stages were plotted on to it, and from it the whole structure of the molecule could be derived by the reverse transformation from reciprocal into real space. The difficulty of course is that the amplitudes of the reflexions are unknown—to derive them from the measured intensities demands a knowledge of their phase angles, which are not susceptible of direct measurement.

To overcome this difficulty use was made of a theoretical result which has been noticed on several occasions.[53], [54] It states in effect that the finite size of the molecules produces a recognizable impress on the diffraction pattern by imposing a calculable character on the Fourier transform. In the form which is useful in the present context it can be thought of as a consequence of standard optical diffraction theory. The diffraction pattern of a slit consists of a central maximum with positive phase, surrounded by a set of equidistant fringes whose phases are alternatively positive and negative. The separation of these fringes is a function of the width of the slit. Now if the slit is replaced by a complicated diffracting object of the same overall width, the fringes no longer have equal widths, nor do their amplitudes decrease regularly with increasing angle of diffraction. However the *average* wavelength of the fringes remains the same as those of the simple slit, and is set only by the overall width of the object. Further, there is a limit to the rapidity with which the transform can oscillate from positive to negative, for it may be regarded as the superposition of a large number of sinusoidal waves produced by the interference of radiation scattered from all possible pairs of points in the object: the wave of shortest wavelength is derived from the pair of points which are furthest apart, i.e. at the outer confines of the object: and the rate of oscillation of the transform as a whole cannot exceed that of the component of shortest

* In the general case two maps would be required, one for the real part and one for the imaginary part of the transform. In the example discussed below the part of the transform used is everywhere real, so that one map only is required.

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wavelength.* Thus an independent knowledge of the dimensions of the object—in the present case, the haemoglobin molecule—makes it possible to predict the maximum rate of oscillation of the Fourier transform.

Now in the slit, and also in the *b* projection of a crystal of space group *C*2 (i.e. the *hOl* reflexions), there is a centre of symmetry; this means that the transform must be everywhere real, or in other words the X-ray reflexions must have phase angles 0 or π—thus the fringes must be alternately positive and negative and must be separated by nodes of zero amplitude. Our theoretical result tells us that these nodes must be

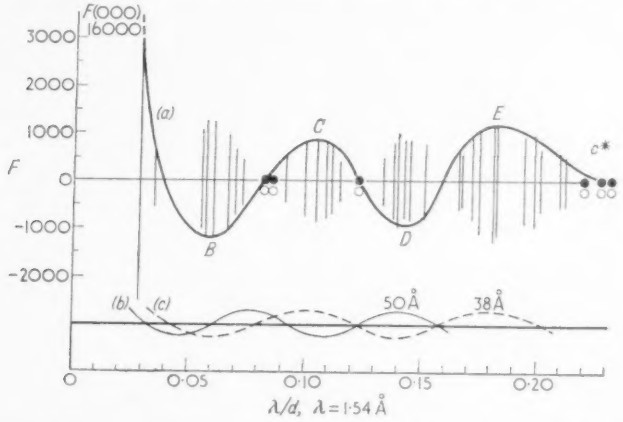

Fig. 11. (*a*) Corrected values of (*OOl*) reflexions of horse haemoglobin. (*b*) (*c*) Positions of maxima and minima in diffraction patterns of molecules respectively 50 Å and 30 Å in width. For explanation see text (after BRAGG and PERUTZ[52])

at least a certain minimum distance apart, set by the dimensions of the slit (or molecule), a result which holds good whatever the internal structure may be*; the regions between these nodes must be alternately positive and negative.

So far the method has been applied only to the *OOl* reflexions of haemoglobin. The "slit width" is taken to be of the order of 50 Å (the overall dimension of the hydrated molecule in the *b* direction, derived from the two earlier studies, being 55 Å). In Fig. 11 the amplitude of the *OOl* reflexions from all the available shrinkage stages are plotted as a function of reciprocal spacing: the lines representing the amplitudes are drawn both above and below the axis, to indicate that their signs are initially unknown, and small or absent reflexions are indicated by

* These statements are not strictly true in all cases; but they hold in sufficiently good approximation to provide valuable criteria for sign determination. Generally, however, some ambiguities and errors will remain and must be removed by other means.

circles. The curve has been drawn in reference to the principles indicated above: namely that positive and negative regions must alternate and be separated by nodes, and that the wavelengths of the swings of the transform must correspond with the width of the molecule (maxima and minima for slits of 38 Å and 50 Å are indicated beneath). The signs of regions *B*, *C* and *D* seem to be settled unambigously as −, +, − on the basis of the first principle alone; and the minimum wavelength indicates with little doubt that there is another node between *E* and *F* (here no direct check is possible since no reciprocal lattice point happens to occur at the interesting point); thus region *E* is positive and *F* is negative. The signs of the reflexions are now determined, and a Fourier synthesis

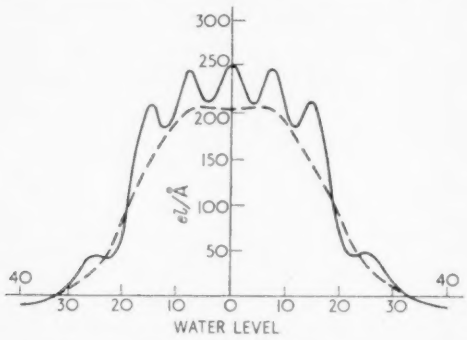

Fig. 12. Electron density of haemoglobin molecule, projected on *c**-axis. For explanation see text (after BRAGG and PERUTZ[52])

of any shrinkage stage can be computed, giving the projected electron density along the *c**-axis. This is shown by the full line of Fig. 12; the dotted line is the result of a similar computation using values of $|F(water)| - |F(salt)|$, i.e. a projection of the "molecule" of uniform electron density which was discussed above (p. 267).

The relation between this result and those earlier discussed can be seen by comparing it with the "end-on" model illustrated in Fig. 8*a*: the 1-dimensional projection is on to the *c**-axis, i.e. perpendicular to the five layers of the earlier model, and like it has five maxima of density. The agreement between the two results is most interesting and suggests that the structure of the molecule is in some way based on five layers, though it must be remembered that the fluctuations in electron density given by the full curve of Fig. 12 are far smaller than would be expected if the molecule consisted of just five layers of polypeptide chains, one on top of the other. At the present stage it is very easy to take too simple a view of the structure of the haemoglobin molecule.

These results, important though they may be, are only the beginning of the application of the method. The next step should be to extend

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it to two dimensions, in the *b* projection which, being centrosymmetrical, can be derived from reflexions (the *hOl*'s) whose phases are all 0 or π. It is thought by the authors of the paper just described[52] that this extension is possible, though not straightforward. The difficulty about it is that haemoglobin crystals shrink in such a way that *a* remains constant: in other words the layer-lines of the reciprocal lattice remain a constant distance apart. This distance is so great that the criterion of minimum wavelength cannot be applied to establish the relative signs of successive layer-lines (though of course the relative signs *within* each layer line should be determinable by the same procedure as that described above for the zero layer): it may however be possible to bridge the gaps by studying other forms of haemoglobin in which the molecules are differently oriented relative to the main shrinkage direction.

It is not at present clear how it will be possible to extend the method further, to three dimensions; the general reflexions *hkl* may have any phase, so that although the real and imaginary parts of the transform must each satisfy the wavelength criterion and exhibit nodes as described above, there are not necessarily nodes of zero intensity in the X-ray pattern, since nodes in the real part and in the imaginary part do not necessarily coincide. There is the same difficulty about the other two projections of monoclinic horse haemoglobin; both the *Okl*'s and the *hkO*'s may have general phase. Probably orthorhombic crystals will be easier to deal with, since they have three real projections (i.e. *hkO*, *Okl*, and *hOl* are all restricted to phases of 0 or π).

A most promising step forward in the analysis of the structure of haemoglobin has recently been made by PERUTZ and his collaborators[99]. It has been found possible to attach mercury atoms, two in each haemoglobin molecule, to the free —SH groups of cysteine, and to crystallize the resulting mercury derivative. The crystals prove to be exactly isomorphous with those of unsubstituted haemoglobin, and while the general distribution of intensities in the diffraction pattern is more or less the same, many of the reflexions are significantly altered. This makes it possible to apply the "heavy atom" method often used with success in classical crystallography, and thus to determine unambiguously the signs of those reflexions whose intensities are significantly changed, at any rate if their phases are real (*i.e.* in haemoglobin the *hOl* reflexions). By computing a "difference Patterson", using values of $(|F_{Hg}| - |F_{normal}|)^2$ as terms of the series, the Hg–Hg vectors can be established; the actual positions of the mercury atoms in the unit cell can then be found within 1 Å by straightforward methods. Next the contributions, positive or negative, of the mercury atoms to the altered reflexions are computed; and hence, by observing whether a given reflexion has become stronger or weaker, its sign can immediately be

found, being respectively equal or opposite to that of the mercury contribution. The signs so determined are used to establish unambiguously the run of the nodes and loops in the layer lines of the Fourier transform of the *b* projection, after which it is merely a matter of computation to derive an electron density projection of the unit cell. At the present stage of the analysis this projection includes only reflexions of spacing greater than 7Å, and is thus too coarse to reveal many significant features of the structure. However these seems no reason in principle why the method cannot be extended to give a more highly resolved projection; and it has the great advantage over all others that it is direct and does not involve guess-work.

Other methods

The methods and hypotheses so far described represent the development of what might be called the classical school of protein crystallography, in the sense that they are the tools and preconceptions of the majority of workers in the field. However in a subject so immature as this one it is natural and fortunate that views are also expressed which lie right outside the main stream of its development. Such unconventional standpoints certainly cannot be rejected out of hand, since there is still hardly any generally agreed and conclusively established result in the whole subject, even in its most "classical" reaches.

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The most fully developed of these other approaches is the globulite theory of WRINCH,[55] which is a revised version of the cyclol hypothesis earlier proposed by the same author.[56] In essence the hypothesis is that proteins contain some kind of basic sub-unit of a regular polygonal, cage-like form, known as a "globulite"; its diameter might be between 9 and 15 Å and its molecular weight 5–6000.[57] Several such globulites would make up the whole molecule; e.g. three for myoglobin, twelve for haemoglobin.* The validity of the hypothesis is examined by computing the Fourier transform and Patterson synthesis of the proposed globulite,[58], [59] and comparing it with the features of the pattern actually observed. The role of water molecules, attached in a highly ordered way to the globulites, has been stressed, being used to explain the shell of high vector density at 5 Å in the haemoglobin and ribonuclease three-dimensional Patterson syntheses.[34] The polypeptide chain as such almost fades from the picture: instead of the essentially polar structures of the classical school we have here basic units of a highly symmetrical character. The development of this theory has led incidentally to a number of valuable theorems concerning the manipulation of Patterson

* The main difference between the new and the old forms of the theory appears to lie in the proposals that the globulites are much smaller than the older cyclols and that a single molecule comprises several of them. In this way several of the difficulties in the old theory (e.g. discrepancies between observed an calculated density) may probably be resolved.

syntheses;[60], [61] but its main conclusions regarding the structure of proteins have not gained the general acceptance of workers in the field. They nevertheless deserve study, particularly in view of the renewed interest in sub-units of protein molecules.

A superficially similar approach, but actually very different in all but name, is that of HARKER, who has proposed[62] that protein molecules may be regarded as made up of "globs" about 10 Å in diameter; each glob is thought of as a single "atom" and the reduced structure, made up of a manageable number of these "atoms", is treated by the methods used in structure determinations of more conventional crystals (e.g. sign determination by means of inequalities). The subdivision into globs is formal: they are not thought of as necessarily having physical individuality, but are used for convenience of mathematical treatment —even their size depends merely on the degree of resolution employed. The validity of such a reduction of the data has been seriously called into question. The virtue claimed for this approach is that it makes no physical assumptions whatever, and is thus not coloured by any of the usual preconceptions; but it is difficult to do it justice in the absence of detailed publications.

Finally—and returning to the classical school—mention should be made of a method which has been outlined by BERNAL.[63], [95] It is in essence a way of rejecting certain combinations of signs of reflexions by taking note of the implications of the physical fact that in the unit cell of a protein viewed at low resolution the variations of electron density must be comparatively slight. It can be shown that the main "features" of the Fourier synthesis of the structure must be produced by the few strongest reflexions: certain sign combinations of these reflexions can be rejected since they would lead to impossibly dense or impossibly tenuous features. The method has not yet been applied exhaustively.

RESULTS

In attempting to summarize the results actually achieved by the x-ray analysis of globular proteins, and to assess their reliability, a reviewer finds himself in a difficult position, particularly if he happens to work in the field himself. The range of creeds held by protein crystallographers covers a complete spectrum from agnosticism to a variety of dogmatic beliefs: this variety is one of its more attractive features, but it is not one which offers any easy possibility of objective appraisal. The present author will do his best, at the inevitable risk of offending some of his more sanguine colleagues by saying too little, some of the less sanguine by saying too much, and his readers by refusing to have complete confidence even in what he does say.

First, then, the evidence for the existence in globular proteins of

parallel sets of polypeptide chains in approximately hexagonal close-packing does seem fairly convincing: we have noted that indications of such a structure have been found in haemoglobin (three species), insulin, myoglobin and ribonuclease. In each case the hexagonal net is slightly distorted, and in each case there is a periodicity of about 5 Å along the chain direction. Having said so much it must be admitted that in some of the proteins the evidence for this structure is very sketchy; it only becomes convincing by the accumulation of instances. It must also be pointed out that in the one protein—haemoglobin—where the matter has been studied quantitatively, it is necessary to hedge about the conclusion with qualifications: that only a part, and probably less than half, of the molecule can exist in this configuration, that the chains even in this part are probably not quite parallel after all. In other words the actual structure must be much more complicated than the generalization suggests, but nevertheless it very probably has as its basis the arrangement of more-or-less parallel chains, more or less in hexagonal packing, which has been described.

As to the configuration of the chains themselves it is impossible to speak with any confidence. For the main part the evidence is of a most indirect kind: that the chains in the globular proteins exhibit—especially in haemoglobin—certain resemblances to those in α-keratin; these are similar to the chains in synthetic polypeptides; and the evidence that the latter are α-helices is strong. On the other hand, the absence of strong 1·5 Å reflexions in ribonuclease, a protein whose diffraction pattern in many respects resembles that of haemoglobin, is disturbing, and indeed a quite different configuration has been proposed for its chains,[17] as we have noticed. To assume that the chain in the globular proteins has the configuration of the α-helix is, in the opinion of the reviewer, the most fruitful working hypothesis to adopt at the present time: but it cannot yet be regarded as proved. In any case the presence of more than one configuration in different parts of the molecule, or in different proteins, is perfectly possible. Fortunately for many purposes it makes no difference what configuration is assumed to be present; in the structure analysis of globular proteins the crucial step is the assumption that lengths of straight chain are present at all—at this stage of the analysis it matters little what configuration they are supposed to assume. When the analysis has gone further the solution to the problem may emerge of its own accord.

Finally we may consider the evidence about the layout of the molecule and its external shape. The only protein about which anything of much general interest can yet be said is haemoglobin. Here we have evidence from two quite different lines of reasoning that the equivalent ellipsoid of the hydrated molecule has dimensions about $55 \times 55 \times 65$ Å; in other words, it is prolate with an axial ratio 1·18. This result is

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encouraging since it agrees well with the values obtained by measurements in solution.[64] It must be emphasized that there is no suggestion that the molecule actually is ellipsoidal; parallelopipeds or cylinders, for example, having the same equivalent ellipticity, would equally fit the facts, and indeed on general grounds an ellipsoid as such would appear to be highly improbable. Next it has been shown that if we look at the molecule along the predominant chain direction (i.e. more or less along the major axis) the chains, approximately in hexagonal close-packing, probably fall into five layers. The model earlier proposed consisted of four layers, but the newer evidence for an odd rather than an even number is very strong. That the number is five (rather than three or seven) is very probable, though not so clearly demonstrated as that it is odd. There are, however, still difficulties of a numerological kind about haemoglobin: (*a*) the molecule contains *four* haem groups, and in some species dissociates into *two* sub-units (in at least one species probably into *four*); furthermore the closely-related myoglobin, with its one haem group, has a molecular weight one-*fourth* that of haemoglobin to an accuracy considerably better than 1 per cent (by comparison of iron contents): (*b*) according to the end-group method horse haemoglobin contains *six* separate chains. It is difficult at first glance to see how the same structure could tolerate subdivision into fourths, fifths and sixths. Fortunately the difficulty is not hard to resolve, at least in principle. A molecule consisting of four sub-units can easily appear in projection to have five layers if each sub-unit has more than one layer and if they are fitted together in such a way that they overlap in projection. As to the number of chains, it is possible that there are some uncertainties in the results of the end-group method, and in any case this method does not reveal end-groups which are masked by cyclization of the chain or in other ways. However this is pure speculation, for at present there is no positive information whatever as to the relations of the sub-units (if they exist) to one another or as to the dispositions of the individual chains.

Thus although some features of the haemoglobin molecule now begin to emerge with a certain clarity it is still not possible to propose a model of it which is sufficiently detailed to be of much assistance in other fields. Regarding the other proteins there is even less to be said. The most immediately promising is insulin, partly because of the encouragingly simple Patterson syntheses of its orthorhombic form (see Figs. 4*b*, 5*a*), partly because far more is known of its structure from the chemical point of view than about that of any other protein, as a result of the work of SANGER[65], [66] and others. He has shown that the 12,000 unit to which reference has been made is built up of four polypeptide chains, two containing 21 residues each and two containing 30 each; he has also worked out the complete sequence of amino-acid

TABLE

Unit cells and space groups of proteins

Protein	*Space group*	*Cell dimensions*				*Mols/cell*	M. Wt $\times 10^{-3}$	*Patterson synthesis no. of dimensions*	*References*
		a	*b*	*c*	*β*				
Trypsin inhibitor	$C6_32$	Wet 111		122		64?	9		(67)
Trypsin inhibitor, compound with trypsin	A2	Wet 113	123	75	139°	8–12	30		(67)
Insulin I	R3	Wet 83·0		34·0		3	12	3	(13), (30)
		Dry 74·8		30·9				2	
Insulin II	$P2_12_12_1$	Wet 49·2	57·6	39·5		4		2	(14)
		Dry 44	51·4	30·4				3	
Ribonuclease I	$P2_1$	Wet 30·90	38·80	54·06	106·0°	2	13	3	(3)
		Dry 29·10	30·08	51·03	114·0°				
Ribonuclease II	$P2_12_12_1$	Dry 36·6	40·5	52·3		4			(68)
Lysozyme chloride I	$P4_12_1$	Wet 79·1		37·9		8	13·9		(69)
		Dry 71·2		31·4				3	
Lysozyme chloride II	$P2_12_12_1$ or $P2_12_12$	Wet 56·3	65·2	30·6		4			(70)
Lysozyme nitrate, iodide	$P2_1$	Wet 27·9	63	66·3	114·25°	4			(67)
Myoglobin, horse I	$P2_1$	Wet 57·3	30·8	57·0	112°	2	17	2	(20)
		Dry 51·5	28·0	37·0	100°				
Myoglobin, horse II	$P2_1(\psi-P22_12)$	Wet 57·0	30·8	106·0	91°	4		2	(35)
		Dry 53·5	28·8	84·5	c. 90°				
Myoglobin, finback whale	$P2_12_12$	Wet 97·4	39·8	42·5		4		2	(71)
Myoglobin, blue whale I	$P2_12_12_1$	Wet 144·5	37·7	107·5		12–16		2	(72)

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Myoglobin, blue whale II	$P2_12_12_1$	Wet	33·9	60·4	76·6		4		2	(100)
Myoglobin, sperm whale I*	$P2_1$	Wet	65 7	31·1	34·8	105·5°	2		3	(73)
		Dry	61·8	26·9	33·9	105·5°	2			
Myoglobin, sperm whale II	$P2_12_12_1$	Wet	48·9	40·2	79·3		4		2	(100)
Myoglobin, penguin I	$C222_1$	Wet	48·29	80·22	78·47		8		2	(100)
		Dry	43·18	70·78	77·10					
Myoglobin, penguin II (imidazole)	$P2_12_12_1$	Wet	106·4	39·10	45·34		4		2	(100)
Myoglobin, penguin III (imidazole)	$P2_12_12_1$ (?)	Wet	94·5	43·5	38·7		4			(100)
Myoglobin, seal I	$P2_1$	Wet	57·8	29·69	57·1	112°	2			(100)
Myoglobin, seal II	A2	Wet	57·74	29·61	106·2	102°	4		2	(100)
Chymotrypsin	$P2_1$	Wet	49·6	67·8	66·5	102°	4?	21·5		(74)
		Dry	45	62·5	57·5	112°				
γ-chymotrypsin	$P4_32_1$	Wet	69·5		97·5		8			(75)
		Dry	63·1		74·5					
Pepsin	$C6_12$	Wet	67·9		292		12	36	2	(76), (77)
		Dry	60·5		268					
β-lactoglobulin I	$P2_12_12_1$	Wet	69·29	70·42	156·5		8	35	2	(18), (78)
		Dry	60·7	61·0	112·4					
β-lactoglobulin II	$P4_32_1$	Wet	67·5		135·5		8			(79), (80)
		Dry	56		130					
Haemoglobin, horse (met-, CO-, oxy-) I	C2	Wet	109	63·2	54·4	111°	2	66·7	3	(47), (50), (81)
		Dry	102	56	49	134°				
Haemoglobin, horse (met-, CO-, oxy-) II	$P2_12_12_1$	Wet	122	82·4	63·7		4			(47)
		Dry	95	72	61					
Haemoglobin, horse (met-, CO-, oxy-) III	P2	Wet	64·5	101	104	102°	4			(77)

* Crystals of this form have also been obtained from common porpoise, mink whale, sei whale, and humpback whale.

TABLE

Unit cells and space groups of proteins (*contd.*)

Protein	*Space group*	*Cell dimensions*				*Mols/cell*	M. Wt $\times 10^{-3}$	*Patterson synthesis no. of dimensions*	*References*
		a	*b*	*c*	*β*				
Haemoglobin, horse (reduced)	$C3_12$	Wet 56·1 Dry 47·4		354 308		6			(77)
Haemoglobin, sheep (adult)	C2	Wet 164	70	66	94·5°	4			(82)
Haemoglobin, sheep (foetal) I	$B22_12$	Wet 112 Dry 78	108 99	56 54		4			(82)
Haemoglobin, sheep (foetal) II	$P2_1$	Wet 55 Dry 48	83 71	64 58	102° 101°	2			(82)
Haemoglobin, human (met-, CO-, oxy-) I	$P2_12_12_1$	Wet 85·2 Dry 70·4	77·0 73·3	86·0 77·0		4			(83)
Haemoglobin, human (met-, CO-, oxy-) II	$P2_12_12_1$?	Dry 50	55	123		4			(77)
Haemoglobin, human (met-, CO-, oxy-) III	$P4_12_1$	Wet 53·7 Dry 47·4		193·5 174		4		2	(84)
Haemoglobin, human (reduced) I	$P2_1$	Wet 66	98	110	98°	4			(77)
Haemoglobin, human (reduced) II	$P2_1$	Wet 62·5 Dry 59	83·2 70	52·8 47·5	98° 97·5°	2		2	(31)
Haemoglobin, human (reduced) III	$P2_1$	Wet 64·8	97·5	108·1	114·8°	4		2	(85)
Haemoglobin, human (in sickle-cell anaemia)	$P2_12_12$	Wet 202 Dry 181	107 91·5	53·2 44·0		8			(84)

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Haemoglobin, human foetal	(tetrag.)	Wet 198				101(?)	16?		(86)
Haemoglobin, hamster	$P2_12_12_1$	Wet 123	88	60			4		(87)
Haemoglobin, ox	$P2_12_12_1$	Wet 64·6	160	55·2			4		(32)
		Dry 61	137	50					
Serum albumin, horse	(hexag.)	Wet 96·7		145			6	70–73	(75)
		Dry 74·5		130					
Serum albumin, human	C2	Wet 178	54	166	91°		8	65	(4)
		Dry 168	38	134	95°				
Serum albumin—mercaptalbumin mercury dimer	$P2_12_12$	Wet 165	83	63			2	131·5	(4)
		Dry 148	50	51·5					
Excelsin	R3	Dry 85·5			60·5°		1	294	(88)
Tobacco seed globulin	F(cubic)	Dry 123					4	300	(89)
Edestin / Squash seed globulin	F(cubic)	Dry 140					4	c. 300	(90)
Ferritin	$P2_12_12_1$	Wet 131·5	131·5	186			4	460	(2)
		Dry 109	109	154					
Apoferritin	F(cubic)?	Wet 186					8		(91)
					α	β	γ		
Tobacco necrosis protein	Pl	Wet 179*	176*	179*	83°	109°	119°	1	1800	(92)
		Dry 157*	154*	147*	100°	110°	120°			
Turnip yellow virus	F(cubic)	Wet 706					8	3500	(93)
		Dry 528							
Turnip yellow virus (nucleic acid free)	F(cubic)	Wet 725					8		(93)
		Dry 550							
Tomato bushy stunt virus	I(cubic)	Wet 386					2	10600	(94)
		Dry 314							

* Packing unit–not unit cell.

residues in each chain. The chains are linked by six –S–S– bridges in ways still to be determined: but if the detailed arrangement of cross-links can be found by chemical means it is obvious that the possibilities for arranging the four chains in space will be greatly narrowed down. From the crystallographic point of view a tentative model has been proposed[44] which has been illustrated in Fig. 8*c*. This consists of four parallel lengths of chain and as far as can at present be seen it does not conflict with the chemical evidence; it probably can also be made to fit the rhombohedral unit cell.[28]

An illustration has been given (Fig. 8*b*) of a plausible model of the ribonuclease molecule, deduced from the end-on Patterson projection: in view of the intensive study of the three-dimensional Patterson synthesis now in progress, this model should probably be regarded as merely provisional. The same can be said of models which have been proposed for the structure of myoglobin.[20] In the case of lysozyme no definite model has yet been proposed.[16]

In conclusion it must be stressed that X-ray studies of proteins are complementary to, rather than rivals of, researches into protein structure conducted by biochemists and organic chemists. The refined chemical techniques, which have given us virtually complete amino-acid balance sheets of many proteins, and which are now beginning to give useful information about the order of residues in the chains, work at a level of detail which X-ray analysis cannot attain; not only are its methods of interpretation inadequate, but the diffraction pattern hardly extends to the resolution necessary to separate individual atoms—and to imagine these difficulties overcome involves a vast extrapolation from our present day powers and techniques. On the other hand there is good hope that X-ray methods may give indications of the general layout of the molecule, of its architecture as a three-dimensional object; and in spite of the triumphs of stereochemistry, leading for example to the elucidation by purely chemical means of the steric arrangement of the residues in molecules as complex as the carbohydrates and the sterols, it seems to be an equally vast extrapolation from present-day stereochemical techniques to suppose that they can be developed to the point required to elucidate unaided the spatial architecture of a protein molecule. Nor does any other technique with the exception of X-ray crystallography, offer hopes of solving this kind of problem.

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FACTS AND THEORIES ABOUT MUSCLE

D. R. Wilkie

Facts and theories are natural enemies. A theory may succeed for a time in domesticating some facts, but sooner or later inevitably the facts revert to their predatory ways. Theories deserve our sympathy, for they are indispensable in the development of science. They systematize, exposing relationship between facts that seemed unrelated; they establish a scale of values among facts, showing one to be more important than another; they enable us to extrapolate from the known to the unknown, to predict the results of experiments not yet performed; and they suggest which new experiments may be worth attempting. However, theories are dangerous too, for they often function as blinkers instead of spectacles. Misplaced confidence in a theory can effectively prevent us from seeing facts as they really are.

This review begins with a summary of the properties of living muscle. Our knowledge of these properties has increased greatly in recent years, largely through the work of A. V. Hill; and any theory about the mechanism of muscular contraction must take account of this new knowledge.

Several theories of contraction are next described and analysed, though no attempt is made to give a final verdict about any of them. It is often difficult to assess the value of a scientific theory, and the review ends with an account of some of the factors to be borne in mind when making such an evaluation.

Living Muscle

The account which will be given here of the properties of living muscle must be short; it cannot therefore be complete. Attention will be concentrated on those features of muscle behaviour which have given rise to theoretical speculations about the contractile mechanism.

All muscles do not have the same properties (though the similarities are far more striking than the differences) and since striped muscles from the frog are easily obtained, maintained and stimulated outside the body, much experimental work has been done with them. The working of the muscle is almost certainly not affected by its removal from the animal body.[1] In an oxygenated solution of suitable ionic composition a thin muscle can easily obtain an adequate supply of oxygen, particularly at low temperature; while the muscle's glycogen content represents an enormous store of potential energy. Under

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suitable conditions, and protected from bacterial attack, isolated frog muscle can survive for three weeks, even at room temperature.[2] The length of time during which its mechanical and thermal responses remain consistent depends largely on the amount of stimulation it receives. Perfectly consistent twitches may be obtained for at least two days after dissection, though more rapid deterioration follows tetanic stimulation, at least under isometric conditions.[3], [4]

The onset of contraction

The physical changes which constitute muscular contraction begin sharply very soon after stimulation. However, the velocity with which

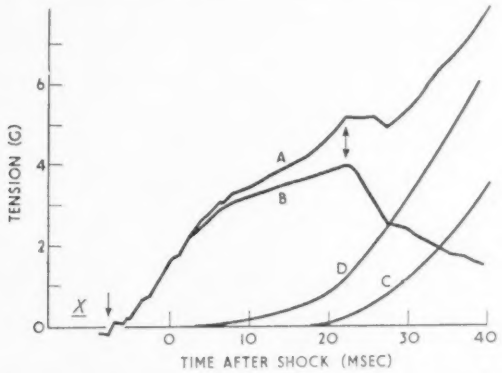

Fig. 1. The effect of a stretch during the latent period on the tension developed by a frog's semimembranosus at 0°C. Stimulus at time zero. Stretch applied during period between arrows. (*A*) Stretch with stimulus. (*B*) Stretch without stimulus. (*C*) Isometric tension development. (*D*) Difference between *A* and *B*, i.e. extra resistance to stretch resulting from activity of muscle (from HILL, A. V.; *Proc. Roy. Soc.* (B) 138 (1951) 343)

the wave of excitation is conducted down a muscle fibre is not very great (30–40 cm/sec. at 0°C) compared with the length of the muscle (e.g. 3 cm for a frog's sartorius) so the sharpness of onset is ordinarily blurred out; for different parts along the length of the muscle start their activity at different times.[5] In many investigations it is possible to reduce this difficulty considerably by stimulating the muscle at many points simultaneously on a multi-electrode assembly.[6] All the details given here relate to frog's muscle at 0°C.

Resistance to stretch

It then becomes possible to detect a change in the mechanical condition of the muscle within 5 msec. of stimulation; so early in fact that the action potential has not yet passed its peak.[7] The change is shown up

as an increased resistance to imposed stretching (Fig. 1) coming on about 3 msec. after stimulation.

Isometric tension development

Spontaneous change in isometric tension cannot be detected until about 17 msec after stimulation (Fig. 1, curve *C*) unless a tension recorder of much higher sensitivity is employed, for example, a piezo-electric isometric lever. With this sensitive instrument (compare ordinate scales of Figs. 1 and 2) the onset of contraction is seen to be diphasic; [8], [9]

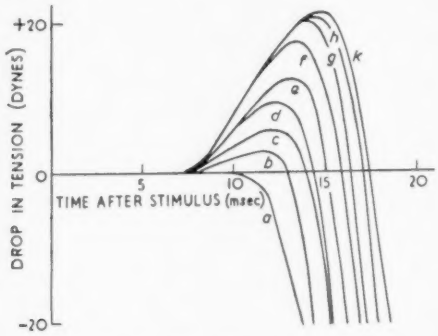

Fig. 2. Early tension changes during isometric twitches of frog sartorius at 0°C at various muscle lengths, in millimetres; (*a*) 27, (*b*) 30, (*c*) 31, (*d*) 32, (*e*) 33, (*f*) 34, (*g*) 35, (*h*) 36, (*k*) 37. Length in body 30·5 mm. Fall in tension shown as an upward deflection (from ABBOTT, B. C. and RITCHIE, J. M.; *J. Physiol.* 113 (1951) 333)

for if the muscle is initially exerting a slight resting tension (e.g. curve *e*, Fig. 2) the tension falls and then rises again, passing its original value at about 15 msec. Both the initial fall in tension ("latency relaxation") and the delay before positive tension is developed, are greater the greater the initial length (Fig. 2). The interesting thing about latency relaxation is that by the time the tension begins to fall increased resistance to stretch is already well developed. The fall in tension can only mean, therefore, that at some point in the muscle active lengthening has taken place.

HILL[7] has pointed out that the shape of the latency relaxation curves at different initial tensions can be explained by supposing that the initial lengthening takes place not in the contractile part of the muscle, but in a parallel elastic structure which is also held to be responsible for the tension-length curve of the muscle at rest. The only parallel elastic structure likely to be influenced by stimulation is the sarcolemma; and while there is evidence[10] that in isolated fibres resting tension is borne by the sarcolemma, in whole muscle connective

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tissue between the fibres must bear some of the resting tension, though the relative importance of sarcolemma and connective tissue cannot easily be decided experimentally. *Isotonic shortening* cannot begin until the initial lengthening has been taken up: it is thus not surprising to find no sign of shortening until 15–20 msec after stimulation.[11]

Heat production

Unfortunately heat measurements cannot be made with the muscle on a multi-electrode apparatus, for it would be technically difficult to interpose a large number of electrodes among the thermojunctions

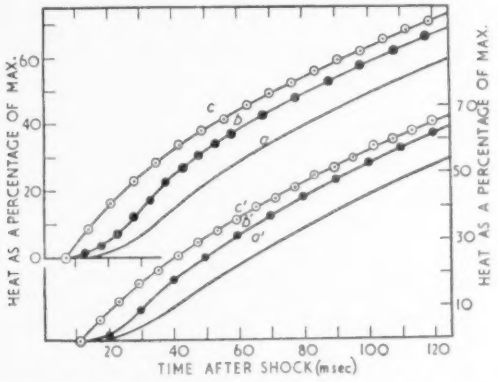

Fig. 3. The early stages of the initial heat production of frog's muscle at 0°C, in an isotonic twitch under small load. (*a*) Experimental heat record (mean of twelve twitches of one muscle); (*b*) corrected for galvanometer lag and heat conduction; (*c*) corrected for time taken in propagation of impulse along the muscle. (*a'*) Experimental heat record (mean from eleven muscles); (*b'*) and (*c'*) are comparable with (*b*) and (*c*) (from HILL, A. V.; *Proc. Roy. Soc.* (B) 136 (1949) 246)

without destroying either their smooth surface or their high insulation resistance. In any case the effect of a finite impulse transmission time can be eliminated mathematically and it then appears[5], [66] that heat production begins suddenly at its full rate about 10 msec after stimulation (Fig. 3).

Twitch and Tetanus

A muscle responds to a single stimulus by giving a twitch, a contraction followed quickly by relaxation. If the stimulus is repeated before the first reponse has had time to die away, the second response becomes fused on to the first. If the stimulus is repeated regularly at a high enough frequency (say 30/sec. in frog muscle at 0°C), individual responses can no longer be detected and the muscle shows a smooth, maintained contraction, or tetanus. Determination of the exact

fusion frequency is to some extent arbitrary, as it depends on the sensitivity of the method employed to detect changes in the muscle.

Is the physical state of the muscle the same during a twitch as it is during a tetanus? On the face of it the answer would seem to be no. Not only are tension development and shortening smaller in a twitch than in a tetanus, but the effect of temperature is quite different in the two cases (rise in temperature makes the twitch tension a good deal smaller, tetanic tension slightly larger). Moreover, the following treatments are known to augment the twitch without much affecting the tetanus—exposure to high hydrostatic pressure[12], adrenaline, caffeine, potassium chloride or a previous tetanus.[13]

Nevertheless, there is strong evidence that the physical state of the muscle *is* the same in the two cases, so that a twitch is nothing but a

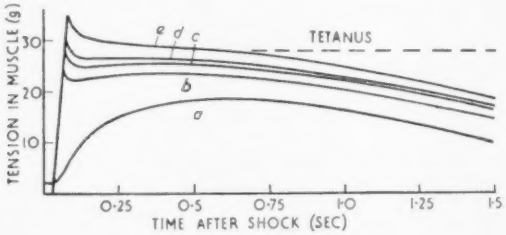

Fig. 4. Effect of stretching a muscle various amounts shortly after a single maximal stimulus. Toad's sartorius 0°C. Final stretched length 36 mm in every case. Broken line, final level of isometric tetanic tension at length 36 mm. After an appropriate stretch (between (*d*) and (*e*)) the tension remains constant and approximately equal to the tetanic tension (from HILL, A. V.; *Proc. Roy. Soc.*, (B) 136 (1949) 405)

tetanus of very short duration. The active state is set up fully and suddenly soon after the stimulus but it is not fully maintained very long (about 45 msec at 0°C, 10 msec. at 20°C),[68] so during much of the twitch the active state is declining. Any experiment to compare twitch and tetanus must therefore be carried out during the initial period whilst activity is at its steady maximum level. There is not time for the full isometric tension to be developed during this short initial period because the active state begins to decay before the contractile part of the muscle has finished stretching the series compliance of the elastic component. (The evidence for the presence of these two components in muscle is reviewed below.) Genuine isometric conditions for the contractile component can be produced experimentally if the tension of the elastic component is raised by giving a stretch of appropriate size after stimulation. When this is done (see Fig. 4) isometric tension development is found to be as large in a twitch as in a tetanus.[6] Moreover, the "genuine" isometric twitch tension revealed by this

technique varies with temperature in roughly the same way as does the tetanic tension, i.e. it varies hardly at all.[14] The large variation in twitch tension with temperature found experimentally comes about simply because the active state is maintained for a much shorter time at the higher temperature. In isotonic experiments the relations between force, speed and heat are found to be exactly the same during the initial part of a twitch and during a tetanus.[15]

The twitch is thus no simple "unit of contraction", and the only advantage to be gained by studying the active state during its transient flight is that, as mentioned earlier, it is easier to do consistent experiments with twitches than with tetani.

The Tension-length Curve

It has been known for very many years that the isometric tension developed by a muscle varies with the length at which the muscle is

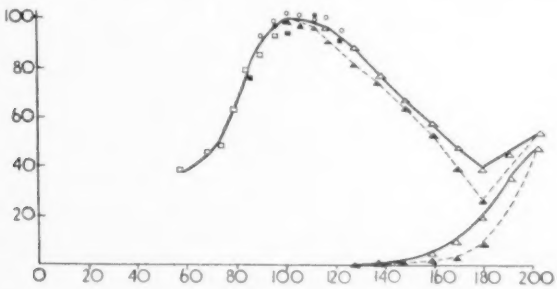

Fig. 5. Tension-length curve of isolated muscle fibre at rest and during tetanic isometric contraction. Ordinate: tension in per cent of maximum. Abscissa: length in per cent of rest-length. Solid lines, ascending order: dotted lines, descending order (from RAMSEY, R. W. and STREET, S. E.; *J. Cell. Comp. Physiol.* 15 (1939) 11)

stimulated, being greatest when the muscle has about the same length that it had in the body and falling off more or less symmetrically at larger and smaller lengths. Since the resting tension contributes to total tension of the stimulated muscle the shape of the curve of total tension against length will depend on the amount of connective tissue in the muscle. Thus single fibre preparations (Fig. 5) in which the resting tension can be attributed to the sarcolemma alone, show a far more pronounced dip at moderate extensions than do whole muscles (Fig. 6).

The relation between tension and length can also be examined in a different way. Instead of altering the length of the resting muscle before stimulation (route B in Fig. 7), the muscle may be stimulated first, then allowed to alter in length (route A, Fig. 7). In the region

below body-length the same curve is drawn by either procedure; but it is not at all certain that this is true if lengths in the shaded region of Fig. 7 are involved.[4]

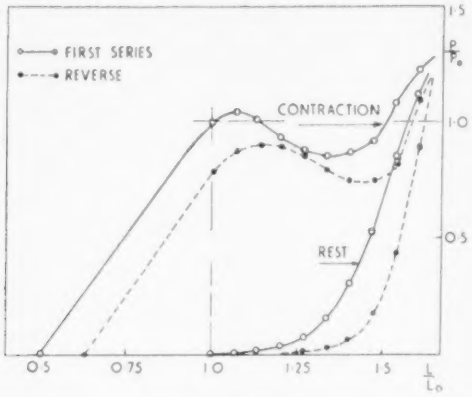

Fig. 6. Tension-length curve of frog's sartorius (0°C) at rest and during tetanic isometric contraction. L_0 = length of muscle in body. P_0 = isometric tension developed at that length (from AUBERT, X., ROQUET, M. L. and VAN DER ELST, J.; *Arch. Intern. de Physiol.* 59 (1951) 240)

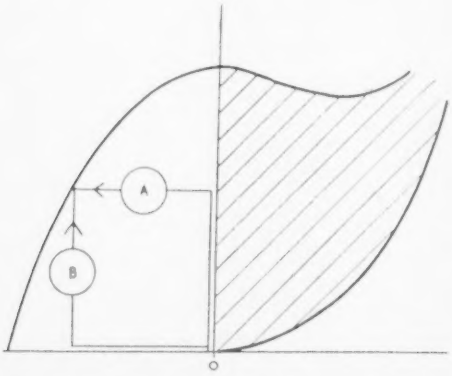

Fig. 7. Diagrammatic tension-length curve of whole muscle. See text (from ABBOTT, B. C. and WILKIE, D. R.; *J. Physiol.* 120 (1953) 214)

The Visco-elastic Theory of Contraction

In the region below body-length the tension-length curve of a contracting muscle resembles the load-extension curve of an elastic body, for in both instances the equilibrium length increases more or less in proportion to the tension.

The basic assumption of the visco-elastic theory was that on stimulation the muscle was actually converted into a new elastic body. If the

stimulation took place with the muscle at about body-length it must, according to the theory, contain potential energy proportional to the area of the tension length curve to the left of its maximum (the unshaded area in Fig. 7). Since this area is approximately triangular, and since an unloaded frog sartorius can shorten down to approximately $2L_0/3$ (where L_0 is the body-length), the potential energy made available should be approximately equal to $P_0L_0/6$ where P_0 is the isometric tension developed at body-length. At extensions beyond body-length, where the tension of the elastic body must fall as its extension increases, the theory runs into difficulties; but these were set on one side. The limited validity of the equation $P_0L_0/6$ ($TL/6$ in more familar symbols), and the assumptions on which it was based, have often been disregarded by physiologists; the equation has been used lightheartedly under all manner of unsuitable circumstances.

According to this theory, if the muscle is allowed to shorten, some of the potential energy is released as external work and the residue degraded to heat at the end of contraction. In isometric contractions, where no external work is done, *all* the potential energy should be turned into heat. The theory was first supported by A. V. HILL's[10] demonstration that in an isometric twitch or short tetanus the heat set free was approximately equal to the energy calculated from the area of the tension-length curve. Difficulties soon arose, however, when experiments were performed on the effect of shortening; for it was found[17] that only a rather small fraction of the theoretical potential energy could actually be realised as external work, even when the shortening was performed "reversibly" against an inertial load instead of a constant force. The advantage of this apparatus was not really that it "opposed the muscle at every instant with a force that it was just able to overcome" since this condition necessarily obtains for any type of shortening; but that, if the inertia was large the muscle was obliged to shorten slowly. The fact that more external work could be done in a slow than in a quick contraction was reasonably explained by supposing that shortening of the new elastic body was opposed by viscous damping. This theory was found to fit accurately the results of experiments on human movement[18], [19]; though the theoretical maximum work extrapolated from the fitted equations was found not to be equal to the area of the human tension-length curve;[20] while the assumption (which was used in deriving the equation) that the force of contraction remained constant throughout a single shortening was not supported by direct measurement; (Ref.[18] in Fig. 5 the slopes of the lines vary over a range of at least 2 : 1).

The first fundamental attack on the visco-elastic theory came in 1924, when FENN[21] showed that when a muscle was allowed to shorten and do work, it produced more heat than it did during an isometric

contraction; not less as the visco-elastic theory had predicted. Nevertheless, linear Maxwellian systems of springs and dashpots were developed, for example by LEVIN and WYMAN[22] to explain the dynamic behaviour of muscle. Even when FENN and MARSH[23] showed in 1935 that in isotonic contraction the relation between applied force and velocity of shortening was not a linear one, the visco-elastic theory might still have been formally saved by making the new assumption that in muscle viscous resistance to shortening was a non-linear function of the velocity. FENN himself thought that the shape of the force-velocity curve was due to some mechanism which regulated the release of energy in muscle. This prediction was strikingly borne out in 1938 by the publication of HILL's classic paper[24], in which FENN's earlier discovery of shortening heat was confirmed and developed through the use of a myothermic technique which had been vastly improved in the interval since 1924.

Heat Production in Muscle

Heat measurements

Everybody knows that muscles produce heat when they are working; and it has been realised for at least a century that the exact relation between the work done and the heat produced during contraction must have a great deal to do with the underlying mechanism by which chemical potential energy is changed into mechanical work. Applied to analogous systems such as steam engines and electric batteries, similar studies have yielded results of the most profound significance.

It is extremely difficult to measure the heat changes in muscle exactly and meaningfully. The maximum rise of temperature associated with a muscle twitch is only about 3×10^{-3}°C, so for accurate measurements the apparatus must be stable enough to resolve in units of a few hundred-thousandths of a degree. Not only are the changes very small, but they take place with a rapid and complex time-course which must be recorded accurately if useful conclusions are to be drawn. Conduction of heat is a rather slow process, so the thermopile must have a very small heat capacity and be in close contact with the muscle. Muscle heat measurements are preyed upon by many artifacts. For example, resting metabolism maintains the temperature of a muscle appreciably above that of the surroundings. If heat loss is not uniform at all points along the muscle, one part will become hotter than another. When the muscle shortens it may draw a hot or cold region into contact with the thermopile and lead to a spurious deflection. A change in the vapour pressure of the muscle will give rise to enormous heat changes;[25] while any mechanical non-uniformity such as local relaxation may cause some of the mechanical energy of contraction to be dissipated as heat inside the muscle. A. V. HILL has developed this

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technique to a remarkable degree of perfection,[26] so that it is now possible to resolve changes to within a few millionths of a degree and a few milliseconds. One result of this improvement in technique has been to shed doubt on the validity of many of the results published before 1938. They should not be accepted without careful scrutiny informed by the new knowledge obtained since their time.[15] The heat produced by a muscle can be divided into three main components: *resting heat* associated with the resting metabolism of the muscle; *initial heat* produced while the muscle is actually contracting and relaxing; and *recovery heat* liberated at a slow rate for the 20–30 minutes following activity.

Resting heat

In resting muscle, as in any other tissue, metabolic changes go on all the time the cells are alive. The net effect of metabolism is that irreversible oxidation of foodstuffs takes place and heat is liberated. Even in the absence of oxygen, metabolism can still proceed for a time (in frog muscle, a few hours; in human brain, a few seconds), but the rate of heat production is reduced; and it appears that intermediate metabolites accumulate, for on re-admitting oxygen the rate of heat production rises for a time above its normal level. The resting heat rate in a frog's sartorius (18°C) is about 2.10^{-4} cal/g-min and presumably this represents the rate of metabolism needed for the ordinary processes of cellular nutrition and maintenance. However in muscle the situation is more complicated than in other tissues, for resting metabolism is linked in some way to the mechanical state of the muscle; both heat production and oxygen consumption are increased several-fold by stretching the muscle.[27] The resting heat rate can also be increased markedly by soaking the muscle in Ringer's fluid containing additional potassium.[28] The high resting heat rate of potassium-treated muscles (unlike that of normal muscles) is sensitive to the pH of the bathing fluid; it passes through a maximum at about $p\mathrm{H} = 7$.[29]

Initial heat

1. *The activation or maintenance heat* is correlated with the presence of the active state. It presumably represents the net thermal effect of chemical processes which have to be kept going all the time the muscle is active. Thus in a twitch the activation heat begins sharply at the same instant as the other signs of activity, rises immediately to its maximum rate, then falls away as the active state begins to decay. In a tetanus the active state is prolonged and the activation heat is correspondingly prolonged. The correlation between the active state and activation heat is not perfect, for the heat rate does fall off somewhat with time during a tetanus even though the active state as

assessed by mechanical criteria does not change.[30] Activation heat also varies with muscle length in a tetanus[24], [30] but not in a twitch.[31]

It is not easy to measure activation heat experimentally, for shortening of the contractile element of the muscle occurs during isometric contraction as a result of stretching of the inert elastic element; the heat record is therefore somewhat contaminated by shortening heat. Probably the best estimate is obtained by stimulating the muscle at such a short length that it neither shortens further nor develops tension.[31]

From the viewpoint of economy, activation heat is energy wasted; for the heat is produced whether work is being done or not. It is the price that must be paid to hold the contractile mechanism in readiness.[51]

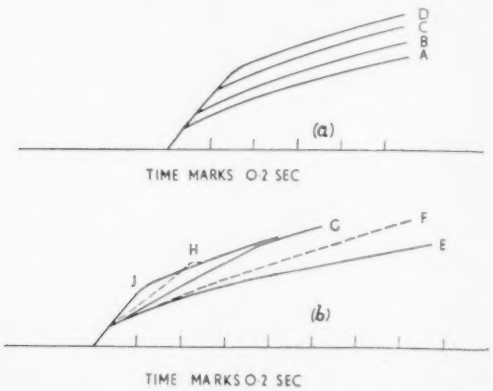

Fig. 8. Heat production during isotonic shortening (tetanus 0°C): (*a*) shortening different distances under constant load of 1·9 g: (*A*) isometric; (*B*) 3·4 mm; (*C*) 6·5 mm; (*D*) 9·6 mm; (*b*) shortening constant distance 6·5 mm under various loads: (*E*) isometric; (*F*) 31·9 g; (*G*) 23·7 g; (*H*) 12·8 g; (*J*) 1·9 g. The ordinate represents total heat produced (from HILL, A. V.; *Proc. Roy. Soc.* (B) 126 (1938) 136)

2. *Shortening heat* appears over and above activation heat whenever shortening of the muscle takes place. Thus, Fig. 8*a* shows total heat produced against time in an isometric tetanus (*A*) and in isotonic shortening (*B*, *C*, *D*). It is clear that extra heat is produced during shortening; moreover, the amount of extra heat (the vertical displacement between curves in Fig. 8*a*) is directly proportional to the distance shortened, see Fig. 9. Fig. 8*b* illustrates the second important property of the shortening heat: the amount of extra heat is independent of the force on the muscle and thus independent of the work done. These results have been amply confirmed since 1938 in experiments on twitches[31] and on tetanic shortening at constant velocity.[30] The proportionality between heat and length remains exact throughout

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shortening of large extent, approaching the maximum shortening which the unloaded muscle can perform.[30] The constant of proportionality a_{thermal} is defined as the amount of shortening heat per centimetre shortening.

3. *Relaxation heat.* It has been known for many years that extra heat is liberated during relaxation. It does not result from any active process, but from the degradation into heat, within the muscle, of mechanical energy which had been generated during contraction, and stored either in a lifted load or in a stretched elastic element. If precautions are taken to prevent this stored energy from being returned to the muscle, no relaxation heat appears.[32]

4. *Initial heat changes during the lengthening of active muscle.* In the course of our everyday lives we use our muscles nearly as often to resist

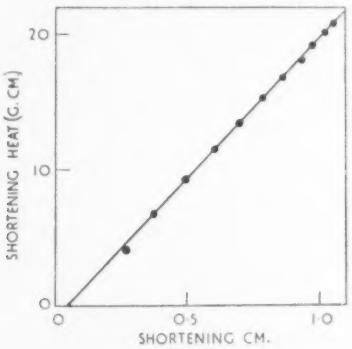

Fig. 9. The relation between distance shortened and shortening heat produced. Frog sartorius 0°C, body-length 32 mm. (from ABBOTT, B. C.; *J. Physiol.* 112 (1951) 438)

movement as to produce it. While resisting, our muscles are active, but are lengthening, not shortening. It is thus of interest to see how isolated muscle behaves under similar conditions, which are just as "normal" as the more commonly observed shortening.

The heat changes during the lengthening of active muscle have been studied experimentally in constant velocity stretches applied to the muscle by a Levin-Wyman ergometer[33] and in isotonic stretches produced by suddenly loading the active muscle with a force greater than its isometric tension.[24] [34] In either case the result is the same: the work done on the muscle does not appear as heat either during activity or during the subsequent relaxation. There is thus a striking difference between the physical state of active and of relaxing muscle, for if the stretch is given during relaxation the work done on the muscle is converted quantitatively into heat. The thermal effect of stretching a resting muscle is different again; for although (for small extensions

of the muscle) heat is given out,[35] the amount of heat is actually greater than the amount of work done on the muscle.[36]

It must be made clear that the absorption of work described above for the case of active muscle can be demonstrated only under rather special conditions. If the force applied to the muscle is too great, a different effect is produced which is described by HILL as "giving" or "irreversible lengthening," when the mechanical work is degraded into

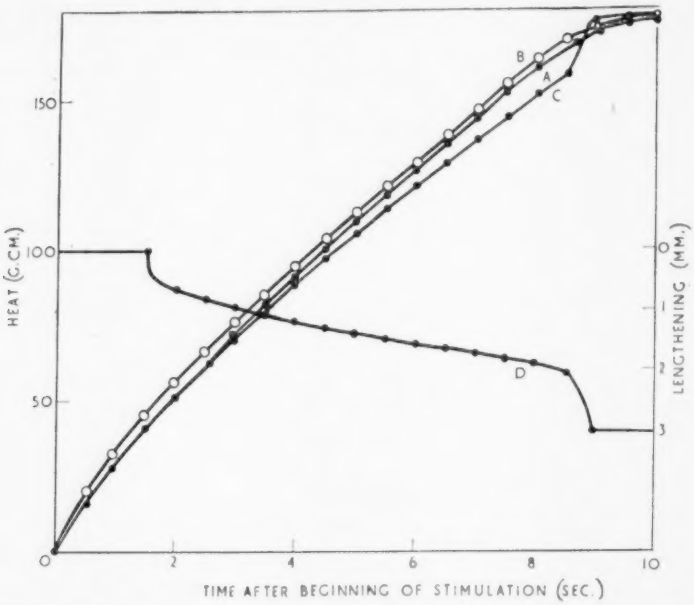

Fig. 10. Heat production during lengthening. (*A*) and (*B*) heat production during isometric contractions. (*C*) heat production during lengthening under 98 g load. (*D*) mechanical record of lengthening (from ABBOTT, B. C. and AUBERT, X.; *Proc. Roy. Soc.* (B) 139 (1951) 104)

heat just as in the case of relaxing muscle. No harm is done to the muscle in the process. Isotonic stretches at tensions up to 1·5 times the maximum isometric tension give the best results for quantitative interpretation in the "reversible" region. As shown in Fig. 10, heat production is actually less during isotonic lengthening (curve *C*) than during isometric contraction at the same length (curves *A* and *B*), in spite of the fact that a large amount of work is being done on the muscle. The total heat deficit is proportional to the amount of lengthening that has taken place. In the corresponding situation with shortening muscle one assumes that the isometric (mainly activation) heat is the result of a process which is not affected by mechanical changes. Making the same assumption here the conclusion to be drawn is that there is a

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negative heat of lengthening corresponding to the heat of shortening (though the constant of proportionality a_{thermal} is up to six times as great in the former as in the latter case); and that work done *on* the muscle, like that done *by* the muscle is without effect on the heat except indirectly through the occurrence of length changes. The assumption that activation and shortening heats reflect independent processes in the muscle is a convenient one, as it leads to a simple picture of the situation; moreover, during shortening the rate of production of shortening heat can actually be varied experimentally over a very wide range without effect on the activation heat. Unfortunately the same cannot be said of lengthening heat, for if an attempt is made to reduce the net heat rate appreciably by rapid stretching, the muscle "gives" and produces more heat, rather than less. This is a pity, for absorption of heat is more interesting than production of heat; cooling, unlike heating, can occur in only a few types of system.

Recovery heat

In resting muscle energy is evidently held available in a very accessible form, for we have seen that it can be released within a few milliseconds of stimulation. The ultimate source of this energy is the oxidation of carbohydrate foodstuffs, but oxidation does not proceed to any appreciable extent during the actual period of activity. It appears that the short-term energy requirement is met from energy production in rapid reactions involving the hydrolysis of adenosine triphosphate and then of creatine phosphate.

Oxidative processes are much slower and the energy they produce is used to "recharge the batteries" by resynthesizing the stores of organic phosphate. Recovery heat is the thermal accompaniment of the recharging process.

The total amount of recovery heat produced by the muscle is approximately equal to its total initial energy production (work + initial heat) under a wide variety of different circumstances.[37] For a fixed duration of stimulus more recovery heat will thus be produced if the muscle has shortened and done work than if it has contracted isometrically. Theoretical interest in the recovery heat arises because heat measurements may be directly correlated with chemical studies into muscle metabolism. For example, agents known to interfere with muscle metabolism also alter the observed pattern of recovery-heat production.[38] When a muscle cannot obtain oxygen it produces energy by hydrolysing glycogen to lactic acid and even when this reaction is prevented by lowering the pH to 6·0 a substantial measure of recovery still takes place. These alterations are reflected by changes in the curve of heat production (Fig. 11, curves A and B respectively). The early

phase of heat absorption has been attributed to the endothermic resynthesis of ATP.

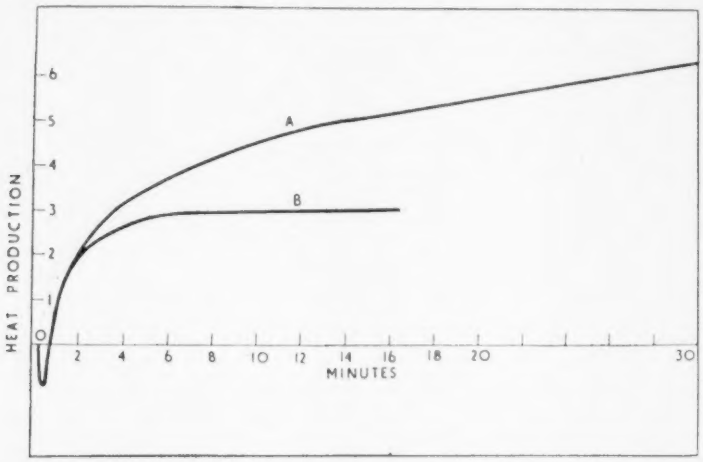

Fig. 11. (*A*) anaerobic delayed heat at *p*H 7·2. (*B*) anaerobic delayed heat under "alactacid" conditions at *p*H 6·0. Heat production is expressed as percentage of initial heat (from HILL, D. K.; *J. Physiol.* 98 (1940) 460)

When oxygen is freely available the muscle produces about twenty times as much recovery heat as it does under anaerobic conditions. The

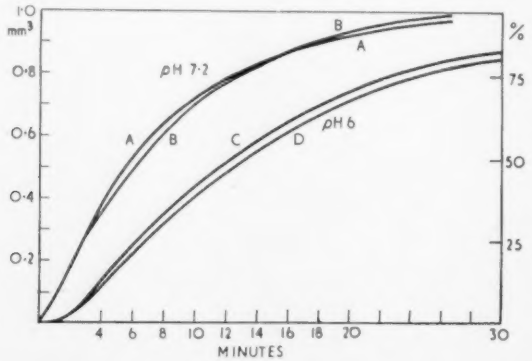

Fig. 12. Comparison of the time courses of oxygen consumption and of oxidative heat production. Ordinates: oxygen uptake mm³; oxidative heat produced expressed as % of initial heat. At *p*H 7·2: (*A*) oxygen; (*B*) heat. At *p*H 6·0: (*C*) heat; (*D*) oxygen (from HILL, D. K.; J. *Physiol*, 98 (1940) 207)

increase is known as the oxidative recovery heat: its time course is strikingly similar to the curve of oxygen uptake, even when metabolism is altered by reducing the *p*H[39] (Fig. 12).

The interpretation of heat changes in muscle

A muscle is a physico-chemical system operating at constant temperature and pressure. What relation exists between heat changes and other changes in such a system? A physico-chemical reaction goes spontaneously only when it can give off free energy by doing so. The free energy may appear as mechanical or electrical work or it may be degraded into heat. However the total heat change accompanying the reaction does not depend only on the amount of free energy degraded in this way but also on the associated entropy change, which may be positive or negative.

$$\text{Heat evolved} = \Delta F + T \,.\, \Delta S - W$$

where ΔF = diminution in free energy of system,

ΔS = diminution in entropy of system,

T = absolute temperature,

W = work done by the system.

If the reaction takes place spontaneously in a test-tube, all the free energy is degraded into heat. Nevertheless, reactions are known in which the entropy term is negative and larger than the free energy term, so that the net effect is an absorption of heat. When no free energy is degraded into heat $\Delta F = W$ and the system is in reversible equilibrium. The only heat change then remaining is that associated with the change in entropy. Under reversible conditions the entropy gained by the environment is just equal to that lost by the system, so the entropy of the universe as a whole is not altered by the change. Whenever free energy is degraded into heat the total entropy of the universe is increased.

These thermodynamic properties must be kept in mind whenever there is a question of inferring the existence of a certain process from measurements of heat and work. Often a clear answer cannot be obtained without utilizing non-thermodynamic results or hypotheses. For example, consider what happens when a piece of rubber is stretched, and when a relaxing muscle is stretched. In both cases an amount of heat is evolved equal to the work done, so there is no change in the internal energy of either system. In the former case heat is evolved because the entropy of the rubber decreases on stretching, and the change is reversed on release. In the latter case no such reversal can be demonstrated experimentally, but this might be because the contractile mechanism is in process of being dismantled. However, one accepts on independent grounds the alternative theory that work is being dissipated irreversibly because it is simpler and because relaxing muscle appears to be viscous rather than elastic in consistency.

In principle it should be possible to interpret the recovery heat by considering the entropies and free energies of the reactions which are believed to go on during recovery. Unfortunately it is seldom possible to do this with certainty, since the thermodynamic quantities are often difficult to measure experimentally. The initial processes are in debt to the final processes to the extent of an unknown amount of free energy. The size of the debt certainly varies with the duration of activity, and with the amount of work done by the muscle. Any discussion of the initial process should take account of the existence of this variable debt, and of the close connection between initial and recovery processes.

Muscular efficiency

How wasteful a process is muscular contraction? In a system at constant temperature waste arises only when the system departs from reversibility. It is thus different from a heat engine, where waste (in the ordinary sense of the word) may be considerable even under reversible conditions. In some chemical systems at constant temperature, for example, storage batteries of low internal resistance, reactions proceed at quite high rates without departing far from reversibility. The "efficiency" of such a system must therefore be a measure of its departure from reversibility if the ordinary meaning of the word "efficiency" is to be respected.

The complex coupled reactions in muscle are not very efficient. Their total effect is the oxidation of glycogen to carbon dioxide and water, and in this reaction the entropy change is known to be small compared with the free energy change. It is therefore justifiable to equate heat evolved with free energy degraded and to estimate the departure from reversibility by the fraction work/(work + heat). For isolated frog muscle[40] and for whole human beings[41] the measured value of the efficiency depends on the exact conditions of working, but in both cases its maximum value is about 20%. The fraction cannot be applied indiscriminately to estimate the efficiency of parts of the contractile cycle (e.g. of the initial processes only) unless their entropy change is known or assumed to be insignificant. Even if conditions are perfectly reversible the fraction is positive, negative or infinite according as the reaction involved is exothermic, endothermic or thermally neutral.

The Dynamics of Shortening

The extra energy produced when a muscle is allowed to shorten consists of two parts—the external work done and the extra shortening heat produced. Thus total extra energy $= (P + a)x$ where $P =$ force on muscle; $a = a_{\text{thermal}}$, the constant of shortening heat; and $x =$ distance shortened. HILL showed by direct experiment[24] that the *rate* at which this extra energy is set free is determined by the force on the muscle.

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In Fig. 13 are shown a number of experimental plots of rate of extra energy production ($= (P + a) \, . \, v$ where $v = dx/dt$, the velocity of shortening) against force. In every case the graph is a straight line which cuts the force axis at a value equal to the isometric tension. Expressing this finding algebraically: $(P + a) \, . \, v = (P_0 - P) \, . \, b$ where P_0 = isometric tension; b = constant.

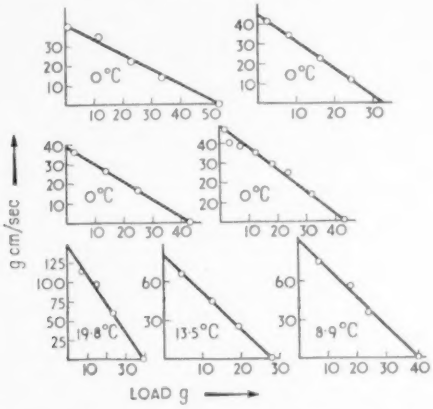

Fig. 13. Several experiments at various temperatures illustrating the relation between the rate of extra energy production, $(P + a)v$; and load P (from HILL, A. V.; *Proc. Roy. Soc.* (B) 126 (1938) 136)

It follows necessarily from this result that the velocity of shortening must itself be a function of the force of contraction, for P and v are the only variables in the equation. Rearranging it into the form

$$(P + a)(v + b) = \text{constant} = (P_0 + a) \, . \, b$$

it can be seen that the curve relating force and velocity is part of a rectangular hyperbola. The demonstration that experimental force-velocity curves can be fitted well by this equation, and that the values of a and b obtained from an empirical curve fitting are the same as those used in calculating Fig. 13 does not contribute any new information; but since a force-velocity curve is concerned directly with easily measured quantities its significance is more readily understood. For this reason the second form of HILL's equation is the more familiar one, and in Fig. 14 are shown direct measurements of force and velocity with a theoretical curve fitted by HILL's equation.

When treated in the way outlined above the experimental results yield up HILL's equation without need for any additional assumptions. However, the situation appears rather more dramatic if one begins by assuming the form of the equation; for the same value of a may then be derived from measurements either of force and velocity or of heat and length.

The characteristic property of contracting muscle is thus seen to be that at every instant the velocity of shortening is uniquely determined by the force on the muscle, just as in an electrical resistance the current is uniquely determined by the voltage. It is as inappropriate to describe the mechanical condition of a contracting muscle in terms of elasticity (a relation between force and length) as it would be to measure an electrical resistance in microfarads.

By applying HILL's equation it became possible, for the first time, to calculate how a contracting muscle would behave under any specified mechanical conditions. Thus the time-course of tension rise in an isometric contraction can be explained as that of a contractile component

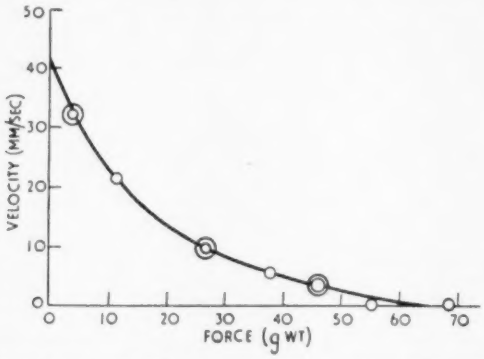

Fig. 14. Isotonic load against initial velocity of shortening. Small circles; experimental points: curve; theoretical, calculated from HILL's equation with $a = 18{\cdot}5$ g wt, $b = 1{\cdot}19$ cm/sec: large circles indicate the points "used up" in curve fitting. Similarity between theory and experiment is significant only at other points on the curve

(obeying HILL's equation) pulling against an elastic element in series with it.[24], [42], [43] At first the tension is small, and the contractile component shortens quickly; but as the elastic element becomes stretched by the rising tension, the rate of shortening becomes less and less, and the tension rises at a progressively declining rate.

In a similar way it is possible to predict what will happen when the muscle pulls against a Levin-Wyman lever;[24] an inertia wheel;[44] a combination of force and inertia;[43] a non-linear compliance[43] (the internal compliance which occurs in muscle is very non-linear); and the combination of force, compliance and inertia which actually confronts a muscle when it is contracting in the living body.[43] All these calculations can be made automatically, if required, by using a simple analogue circuit in which the muscle is represented by a non-linear resistance such as a rectifier; the analogues of mass, compliance and force are then inductance, capacitance and voltage respectively.[45]

HILL's equation is known to describe accurately the force-velocity

relation in a wide variety of different muscles from many species, including frog, toad, cat, tortoise, man, snail, mussel and ray.[4] The variation of the constants from one muscle to another is illustrated by Fig. 15. It is interesting to see that the variation in a/P_0 is small, for this fraction decides the shape of the force-velocity graph (the smaller the fraction the more curved is the graph). The absolute values of a and P_0 vary less in different muscles than do b and V_0. The variation in intrinsic speed is in part a reflexion of the dimensional difference between different animals,[46] in part a reflexion of the varied employment to which muscles are put in the animal body.

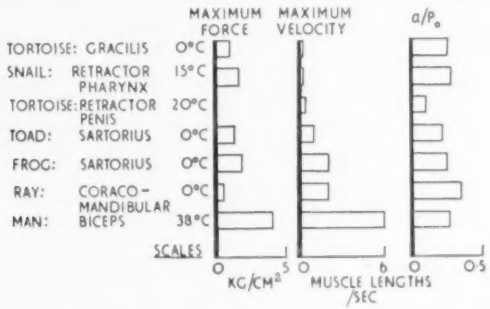

Fig. 15. Muscle constants in different species

The question has sometimes been discussed (e.g. by BUCHTHAL and KAISER[65]) whether the similarity between the thermal and the mechanical estimates of a may not be purely accidental. Their particular objections have been answered,[4] but the general question remains unanswerable until we have a more complete understanding of the contractile process. The two estimates of a are similar not only numerically and dimensionally; but also both have only a small temperature coefficient, and both remain constant throughout shortenings of considerable extent. Admittedly it would be more dramatic if it could be shown that both estimates of a were influenced in the same way by some change in experimental conditions; but if the two values of a are in fact derived from a common process, and if this process is difficult to influence, there is not much one can do further.

Shortening and the tension-length curve

The isometric tension developed by a muscle depends on the length at which the muscle is stimulated, being greatest when the muscle has about the same length that it had in the body. HILL's equation can only legitimately be applied in the region of this maximum, where the variation of P_0 with length is slight: for P_0 appears in the equation as a constant. The v predicted by the equation is always the *initial*

velocity. However, if the value of P_0 is adjusted so that at every instantaneous length l, P_0 is given the appropriate value $(P_0)_l$ indicated on the tension-length curve, HILL's equation can be shown to apply throughout the full extent of shortening.[4] Up to the present the modified HILL's equation

$$(P + a)(v_l + b) = ((P_0)_l + a)b$$

(where v_l is the instantaneous velocity at length l)

has been shown to apply only in the region of the tension-length curve below the length in the body (the unshaded region in Fig. 7). That a and b remain constant at all lengths is interesting because thermal estimates of a also remain constant at all lengths.

The series elastic component of muscle

Since muscles exert their force through tendons it is evident that there must be a series compliance between the muscle and its load (though it is not certain that all the compliance resides in the tendon itself). GASSER and HILL[48] suggested in 1924 that muscle might be a 2-component system; LEVIN and WYMAN[22] incorporated a series elastic component in their Maxwellian model; and HILL showed in 1938[24] how the presence of a series elastic component could explain many of the dynamic properties of muscle. Recent investigations have shown that the elastic element is non-linear, becoming less extensible as the tension is raised. In this property it resembles many other biological materials.

The existence of an independent elastic component has been demonstrated in two ways:

(1) By calculation from the rate of rise of isometric tension.[42, 43] This method rests on the assumption that HILL's equation is accurately obeyed at every instant during contraction. There is a good deal of evidence that this assumption is justified. The method is made more certain by inserting additional known compliances in series with the muscle.[43]

(2) By direct measurement of the relation between tension and length during a release so quick that the contractile component has no time to shorten. So long as the release is quick enough the tension-length curve obtained is independent of the speed of release, i.e. the elastic element is substantially undamped.[47]

Both methods give similar results.

RECENT THEORIES ABOUT MUSCLE

Ramsey's Theory[49]

On examination, this theory is seen to be an extension of the visco-elastic hypothesis in an attempt to explain the new facts reported by

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HILL in 1938. An exactly similar theory has been proposed independently by GASSNER and REICHEL[50] in connection with the elastic properties of resting muscle.

The theory sets out to relate (1) the force-velocity curve, and (2) the shortening heat, to the tension-length curve of active muscle. In order to simplify the mathematics involved, RAMSEY has assumed that the active muscle has a tension-length curve of the idealized shape shown in Fig. 16 ($P_0ABCLL_0DP_0$). Although this does not look much like the tension-length curve of whole muscle (e.g. Fig. 6) it is less

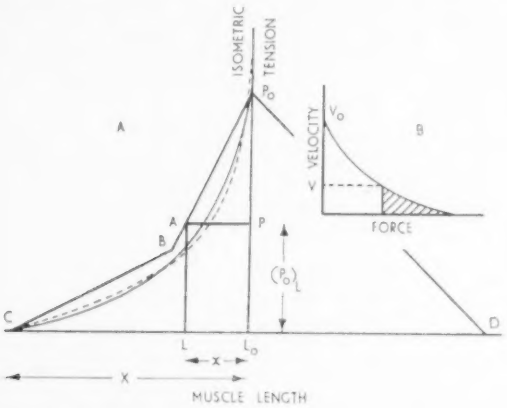

Fig. 16. Figure to illustrate RAMSEY's theory. (*A*) 1. $P_0ABCLL_0DP_0$, idealized tension-length curve. 2. *Full line*, curve drawn from $((P_0)_L + a)(x + b) = a(X + b)$. 3. *Dashed line*, curve drawn from $x = Xe^{-(P_0)_L/a}$. (*B*) Force-velocity curve

different from the corresponding curve for single fibres (Fig. 5). The theory is concerned only with the region below body-length, i.e. the region to the left of L_0 in Fig. 16*A*.

(1) *The force-velocity curve.* RAMSEY assumes that under isotonic conditions the muscle shortens exponentially so that its velocity of shortening at any instant is proportional to the distance it still has to shorten. This is almost exactly the behaviour the muscle would manifest if P_0ABC were the stress-strain curve of an elastic body and if shortening were opposed by a viscous resistance. The initial velocity of shortening against a tension P is therefore proportional to the distance PA; and since this condition applies for all values of P, the force-velocity curve must have the same shape as the tension-length curve. The full line is drawn in from HILL's equation ($a/P_0 = 0{\cdot}25$) for comparison with P_0ABC.

(2) *Shortening heat.* This part of the theory is also based on the assumption that the tension-length curve is similar to an elastic

stress-strain curve. When the muscle shortens from L_0 to L, energy proportional to the area $P_0ALL_0P_0$ is set free. Work proportional to $PALL_0P$ is performed on the load and RAMSEY suggests that the residual energy (proportional to P_0APP_0) is liberated as shortening heat. The larger the shortening, the greater the shortening heat will be. It is quite easy to find a shape for the tension-length curve such that the total shortening heat is exactly a times the distance shortened over the whole range of shortening. This curve, whose equation is $x = Xe^{-(P_0)_L/a}$ is shown in Fig. 16*A* for comparison with curves 1 and 2.

The three curves in Fig. 16*A* are reasonably alike, so RAMSEY's theory is reasonably self-consistent. The similarity between curves 2 and 3 is seen to be very striking indeed when it is realized that the two have been fitted together only by taking common values for a and X. RAMSEY himself used numerical methods to establish his theory, and these did not demonstrate this similarity in so clear a way as the analytical methods used here.

RAMSEY's theory largely succeeds in what it sets out to do, but it suffers from some very serious drawbacks. The tension-length curve of a whole muscle is very different from the idealized theoretical form; and although the *total* amount of heat predicted is proportional to the *total* distance shortened, the theory cannot account for the observation that in living muscle the *rate* of production of shortening heat is also proportional to the *rate* of shortening at any moment. This difficulty can only be circumvented by supposing that elaborate means are provided for "sharing" heat between one moment and another. Finally, RAMSEY's theory will account correctly for the shortening heat only during isotonic shortening, whereas experimental shortening heat is proportional to change in length no matter how much the tension may have varied during the change.

As an extension of the visco-elastic hypothesis RAMSEY's theory may not be altogether successful, but it does draw attention to a previously unrecognized property of the force-velocity curve (see Fig. 16*B*). For all values of a/P_0 between 0·2 and 0·4 the shaded tail area is very nearly proportional to the value of V over the whole range from $V = 0$ to $V = V_0$. In the special case when $a/P_0 = 0{\cdot}25$, the constant of proportionality is exactly equal to a.

The physical significance of this relationship is not clear. In a force-velocity graph, area represents rate-of-working; and it is tempting to link the mathematical demonstration that *tail area* $= a \times V$ with the experimental fact that *shortening heat rate* $= a \times V$.

Polissar's Theory[51]

M. POLISSAR has recently published a comprehensive theory of muscular contraction based on a physico-chemical model.

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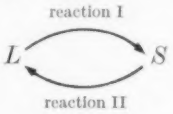
The contractile filaments of active muscle are assumed to consist of minute contractile elements arranged in series along the filament. Immediately after stimulation the units are in a long (L) state, but they can become transformed to a short (S) state by some chemical reaction with their environment (Reaction I, $L \rightarrow S$). By a different chemical reaction, through different intermediate compounds, the reverse change can take place (Reaction II, $S \rightarrow L$). The length of the filament at any given instant depends on the number of units in the L and S state, and therefore on the dynamic equilibrium:



The cycle is driven by a coupled exergonic reaction which is the source of energy for the heat of maintenance in a tetanus and for the mechanical work in an isotonic contraction.

Now in living muscle, shortening is influenced by applied tension: and in POLISSAR's model this effect is brought about by assuming that the relative rate of reactions I and II depends on the tension in the myofilament. Both reactions are assumed to be of first order, i.e.

$$\text{rate of reaction I } (L \rightarrow S) = R_L = k_L[L]$$

$$k_L = \text{rate constant}$$

$$[L] = \text{concentration (or number) of } L \text{ units}$$

The rate constant k_L depends on the temperature T and on the Arrhenius activation energy E (that is the energy barrier which must be exceeded before a potentially active L group can react to become an S group). The relation between these variables is expressed in the familiar equation

$$k_L = Qe^{-E/RT} \ (Q \text{ is a constant})$$

POLISSAR suggests that external tension influences the reaction by altering the value of the activation energy: more specifically, that an external tension W applied to the chain will increase the activation energy from E to $(E + c_L W)$ and thus reduce the rate of reaction I from $k_L[L]$ to $k_L[L] \cdot e^{-c_L W/RT}$. ($c_L$ is another new constant). In an analogous way the rate of reaction II will be increased.

The assumption about the effect of external tension on activation energy is reasonable—one can imagine that the active group has to move a fixed distance before it can react, and that as it is attached to a long-chain polymer, external force can help or hinder its movement. However, reasonableness is a minimal requirement in a scientific theory; and direct experimental evidence about the effect of external force on polymer reactions would be welcome.

The basic model is now complete. Muscle length is a known function of the dynamic equilibrium

$$L \rightleftharpoons S$$

and the state of this equilibrium is a known function of the muscle tension. It is therefore possible to work out, among other things, theoretical relations between force and velocity and between length and tension.

Force-velocity relation

From the above postulates POLISSAR proceeds to derive the equation for the relation between muscle tension and initial velocity of shortening

$$V = \text{const.} \left(A^{1-\frac{W}{W_r}} - B^{\frac{W}{W_r}-1} \right)$$

(V = velocity, W = force, W_r = isometric force, A and B are constants derived from the fundamental constants k_L, c_L etc. given above).

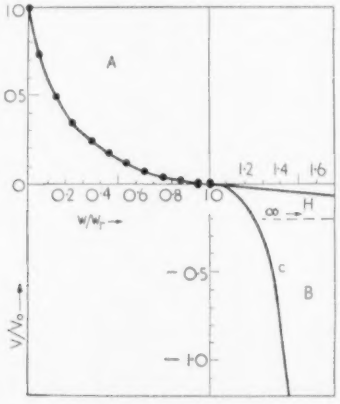


Fig. 17. *Solid curve* shows speed-load relation predicted by the use of physio-chemical model. *Circles* represent experimental values, summarized by HILL's equation. Positive values of v/v_0 represent shortening with loads smaller than W_r; negative values represent lengthening with loads larger than W_r. Curve H is the negative branch of HILL's curve. Dashed line is the asymptote to HILL's curve. Branch c of the curve based on the physio-chemical model is in qualitative agreement with HILL's observations on the "yielding" of the muscle under large loads (from POLISSAR, M. J.; *Amer. J. Physiol.* 168 (1952) 766)

This equation gives a good fit with experimental force-velocity curves (Fig. 17). For negative values of V (muscle lengthening under a load greater than it can bear), POLISSAR's equation gives a closer description of the facts than does HILL's equation, for it predicts that the muscle will yield rapidly if the lengthening force is at all large; and this effect is well-known experimentally as "give" or "slip." However,

the similarity between theory and experiment is not exact, for when the muscle is lengthening the velocity is not a unique function of velocity, as it depends also on time and distance. Since it fits the experimental results, POLISSAR's equation may be used instead of HILL's to calculate, the course of tension rise in an isometric contraction, or the effect of inertia on an isotonic one.

Tension-length curve

The tension-length curve predicted theoretically is shown in Fig. 18*A* curve *ABC*. This curve applies to the contractile component alone;

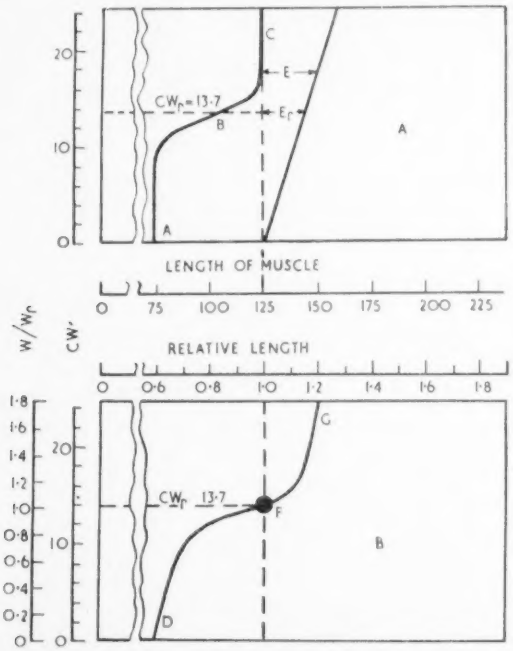


Fig. 18. *Curve ABC*: Theoretical length-tension relation in a model possessing no passive elasticity. *Curve DFG*: theoretical length-tension relation in a model possessing passive elasticity. E_r is the passive elastic stretch at rest-length, at the corresponding tension W_r. *Straight line* in the upper diagram give the stretch E, at any other tension, assuming HOOKE's law. *Curve DFG* is obtained from *curve ABC* by shifting each point of the latter through a horizontal distance equal to the corresponding value of E. *Abscissa;* Length of muscle in arbitrary units (rest-length = 125) and in relative units. *Ordinate*: Tension in arbitrary units ($CW_r = 13{\cdot}7$) and in relative units ($W_r = 1{\cdot}0$) (from POLISSAR, M. J.; *Amer. J. Physiol.* 168 (1952) 766)

but assuming that the series elastic component has the stress-strain curve shown by the straight line in Fig. 18*A*, the tension-length curve for the whole muscle is transformed into curve *DFG*, Fig. 18*B*. This

curve shows little similarity to the tension-length curve as ordinarily determined by adjusting the muscle length prior to stimulation; and POLISSAR states that his theoretical prediction applies only to the tension-length curve obtained in a different way, by tetanizing the muscle and then slowly adjusting it, while still active, to the required length. This latter curve, he claims, has approximately the shape shown in Fig. 18*B*. He quotes in support of this view the results of BLIX[67] (Plate V, Figs. 5*A*, 5*B*, 6*B*). POLLISSAR has unfortunately used GASSER and HILL's old estimate[48] of the compliance of the series elastic element (approximately 12% stretch for full isometric tension) which is now known to be three to four times too big.[47] Even for the whole muscle, therefore, the tension-length curve predicted will be only very slightly different from curve *ABC*. BLIX's curves show a good deal of variation and some fresh experimental evidence would be welcome.

Energy changes

A few simple and reasonable assumptions about the thermal changes accompanying reaction I and II make it possible to predict the heat changes associated with contraction of the model. One result of the cyclic scheme of operations is that a continuous flux of energy is required to maintain tension, exactly as in living muscle. The theory also predicts correctly that velocity of shortening will have a large temperature coefficient and isometric tension development, a small one; it makes possible an ingenious explanation of the "catch" mechanism in slow muscles of high economy. Unfortunately, in other respects the theory disagrees fundamentally with experimental findings.

Thus it incorrectly predicts a positive heat of lengthening instead of the negative one found experimentally[34] (though I understand from POLLISSAR that this fault can be corrected by an appropriate change in the constants): it cannot account for the fact that the amount of extra heat produced per centimetre of shortening is constant, even in shortenings of large extent;[30] and finally the theory gets into serious difficulties when it tries to show how the shortening heat produced could be independent of the work done. The mechanical work done on the load must come from the reaction $L \rightarrow S$, but the theory is worked out as though this reaction produces the same amount of heat *whether it does work or not*. POLLISSAR appears to be aware of this difficulty and tries to circumvent it by supposing that mechanical energy for shortening is somehow stored in the series elastic element. The compliance of this element is now known to be too small for the purpose; and in any case the model, unlike a muscle, would still have the property "more work, less heat". These particular objections need not be fatal to the basic postulates of the theory but it is essential to alter the theory to take account of them; for example,

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by paying closer attention to the energy changes in reaction I and II, which are insufficiently specified by the words "endergonic" and "exergonic." By making clearer the distinction between free energy changes and heat changes it might be possible to preserve the independence between work done and heat produced which can be demonstrated experimentally in living muscle. Thus, the reaction $L \rightarrow S$ might take place under more or less reversible conditions, if chemical means were provided for absorbing any surplus free energy without degrading much of it into heat. This suggestion gains plausibility from the experimental finding that even mechanical work can be stored by muscle as chemical energy.[33] The heat change accompanying the reaction $L \rightarrow S$ is $\Delta F + T\Delta S - W$ (where F = Gibbs' free energy lost by system, S = entropy lost, T = absolute temperature, W = energy lost from the system either as mechanical work or by chemical transfer). According to the suggested hypothesis $\Delta F - W = 0$, so the residue of heat change is entirely associated with the entropy term, $T\Delta S$. ΔS might easily be large, because of the great change in molecular configuration; and it may be positive (i.e. heat given out during the reaction). $T\Delta S$ has the properties required of a shortening heat, being proportional to the distance shortened (i.e. to the amount of reaction), independent of the work done and nearly independent of the temperature.

The Theory of Varga and Szent-Györgyi

This theory[52] was based originally on experiments with muscle models[53], [54] and later extended to explain results obtained from living muscle.[55] The muscle models were prepared from living muscle by extracting it with water or with glycerol solutions. This treatment breaks down the cell walls and removes the crystalloids without, apparently, altering the structure of the protein skeleton. In a suitable ionic medium such models will shorten and develop tension when ATP is added to the bathing solution. This effect is a very specific one, being produced by no other substance except the closely related ITP (inosine triphosphate). Moreover, the models catalyse the hydrolysis of ATP to ADP and thus provoke a continuous release of energy. Maximum tension development and shortening are of about the same absolute magnitude in these models as in living muscle; and the results obtained with them show every sign of being relevant to the interpretation of events in living muscle.[56]

The theory under discussion is based on the assumption that shortening is the result of an all-or-none transformation of molecular shortening units ("autones") from a long L to a short S state. The theory differs from POLISSAR's in supposing that the L and S forms of the autone are in simple reversible equilibrium with one another.

$$L \rightleftharpoons S$$

The final length of the fully shortened muscle depends on the position of this equilibrium; and anything which alters the equilibrium constant of the reaction will alter the amount of shortening.

The theory is immediately confronted by a difficulty. Active living muscle is certainly not in a state of equilibrium, for it produces energy continuously even when it is doing no work: muscle models also liberate energy continuously when they are doing no work. The difficulty can be overcome by making a new assumption, that the observed (non-equilibrium) behaviour is a result of subsidiary effects connected only indirectly with the fundamental contractile process. Having thus disposed

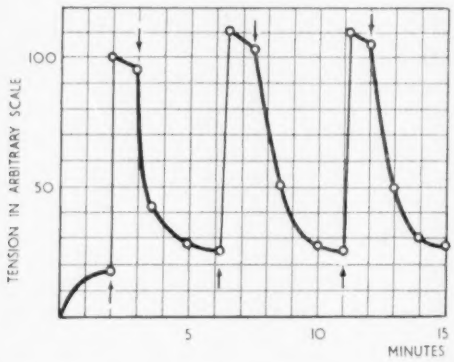

Fig. 19. Isometric tension developed by glycerinated psoas activated by ATP. Lower arrows—muscle transferred 0°C → 15°C. Upper arrors—muscle transferred 15°C → 0°C (from VARGA, L.; *Enzymol.* 14 (1950) 196)

of one crucial test for reversibility it becomes difficult to find other experimental evidence for the theoretical equilibrium. VARGA has shown (Fig. 19) that the maximum tension developed by a muscle model varies reproducibly with temperature and he claims that this constitutes "a flawless demonstration of thermodynamic reversibility."[54] Of course it does not, since many non-equilibrium systems have reproducible properties and are capable of reversal in direction. It is quite possible to apply thermodynamic equation without first establishing the existence of equilibrium conditions; then, if no incompatabilities arise later, to infer that the basic assumptions must have been correct. This is a very uncertain procedure, but it is the only possible one.

According to the theory, the final length of an activated muscle model allowed to shorten freely is determined by the equilibrium constant, K. In general K will vary with the temperature, following the thermodynamic equation

$$\ln K = -\Delta H/RT + \text{constant}$$

(ΔH = heat of reaction, here assumed to be constant over the temperature range considered, T = absolute temperature). K can be estimated experimentally at various temperatures from observation of models shortening at those temperatures; and when $\ln K$ is plotted against $1/T$ a straight line is actually obtained, showing that theory and experiment are compatable (Fig. 20). This agreement by no means proves that the original assumptions were correct, for at least one alternative theory will fit the facts equally well. If the amount of shortening depended on the *rate* of a first-order chemical reaction, rather than on

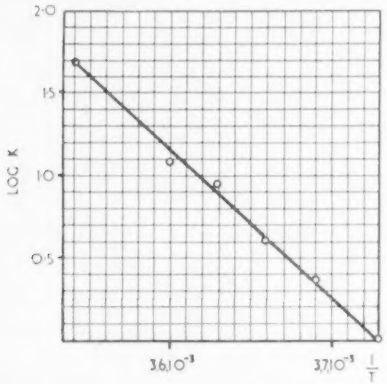

Fig. 20. Graph of $\ln K$ against $1/T$. K calculated from final length of glycerinated muscle allowed to shorten freely in ATP (from VARGA, L.; 14 (1950) 196)

the *position* of a chemical equilibrium, shortening would still be expected to vary with temperature according to the equation:

$$\ln K = -\Delta H_a/RT + \text{constant}$$

though in this case ΔH_a would be the Arrhenius' activation energy. From the experimental evidence one cannot decide between the two theories. In some other ways the second is better, for it avoids the difficulty over reversibility and predicts instead a flux of energy.

Returning to the equilibrium theory; from the experimental values of K one can calculate the standard free energy, ΔF_0 of the reaction $L \rightarrow S$, using the equation

$$\Delta F_0 = -RT\ln K$$

ΔF_0 hardly differs from the free energy released as the reaction moves towards equilibrium,[57] so one can justifiably compare calculated values of ΔF_0 with measurements of the work done by the model at various temperatures. Agreement is fair, as shown in Fig. 21 (compare lower two lines); but unfortunately the comparison rests on an assumed

value for the molecular weight of the autone. (The thermodynamic equations give their answer in energy per mole; the experimental results are in energy per gram.) The figure assumed, 70,000, is to a large degree arbitrary, so the closeness of the lines in Fig. 21 is artificially imposed to the same degree. It is true that there is some independent collateral evidence for the figure 70,000, but similar evidence could probably be brought forward to support some other figure if required.

The equilibrium theory is thus beset by many uncertainties.

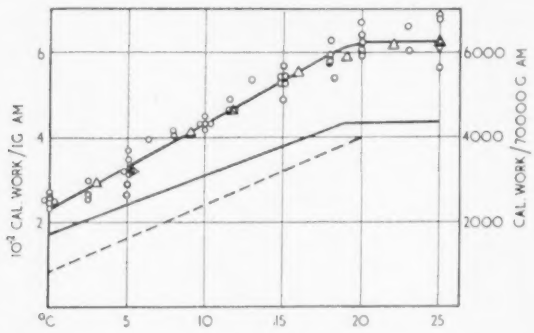

Fig. 21. Variation with temperature of mechanical potential energy per gram of muscle (circles and triangles, left ordinate); and of ΔF_0 per 70,000 g actomyosin (broken line, right ordinate). The middle line shows mechanical potential energy per 70,000 g actomyosin (from VARGA, L.; *Enzymol.* 14 (1950) 196)

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Pryor's Theory[58], [59]

This theory is based on an energy cycle in a rubbery working substance. A description of the theory must be prefaced by a description of rubbery solids in general.

The work done when a perfect gas is compressed isothermally is all turned into heat and conducted away. The internal energy of the gas therefore remains constant but its entropy decreases. The diminution in entropy expresses the fact that the gas is in a less probable state after compression than it was before; that is, if its molecules were left free to distribute themselves at random, they would be even less likely to find themselves by chance in the smaller volume than in the larger one.

Rubbery solids are believed to consist of long chain molecules connected together at only a few points by cross-links. The molecules are thus left free to arrange themselves at random into highly contorted configurations. When the rubber is stretched the chains are forced to straighten out, that is, to assume less probable configurations. Accordingly, the entropy of the rubber decreases and heat is given out, just as in a gas during compression.

The analogy can be taken still further. A gas can be used as the working substance in a heat engine. By taking the gas round a Carnot cycle, heat is conveyed from a high to a low temperature and in the process part of the heat is converted into work. Rubber may be used in an appropriate heat engine in an exactly similar way.[60]

Living muscle, like rubber, is composed of long molecules, but it contains no temperature differences large enough to drive a heat engine. PRYOR's central hypothesis is that certain chemical substances ("plasticizers") alter the elastic properties of rubbery solids in the same way that heat does. Alteration in plasticizer concentration would then have an analogous effect to that produced by alteration in temperature; so the rubbery solid could be taken round a cycle at constant temperature and made to yield work simply by altering the concentration of plasticizer.

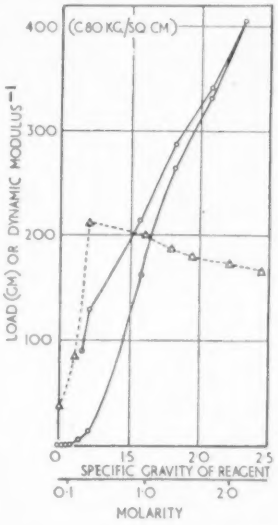

Fig. 22. Full line: relation between isometric tension developed and plasticizer concentration. Broken line: reciprocal of dynamic modulus (from PRYOR, M.; *Progress in Biophysics* 1 (1950) 216)

This is an ingenious idea, but before the theory can be accepted as a possible explanation of muscular contraction, two questions must be answered: (1) do plasticizers actually work in such a way that their concentration may be taken as the analogue of temperature; and (2) does a system working according to the theoretical scheme bear any resemblance to a contracting muscle?

(1) PRYOR attempts to answer the first question experimentally by investigating a model system which consists of a tendon plasticized by mercury-potassium iodide. In this reagent the tendon shortens and becomes rubbery. If the tendon is prevented from shortening it will develop a tension which is more or less proportional to the plasticizer concentration (Fig. 22), and which reaches a maximum absolute value of about 100 Kg/cm². If the plasticizer is washed out of the tendon with distilled water, the tension falls again. An arrangement of tendons can be made to yield work continuously[60] energy being derived from dilution of the plasticizer. This is a striking demonstration, but we are left in doubt about the exact working cycle involved. The situation can only be clarified by an experimental determination of the full set of relations between length, tension and plasticizer concentration (e.g. by measurement of the tension-extension curve at a number of different

plasticizer concentrations. It will then become possible to see exactly how a cyclic change in these variables can be made to yield work.

(2) Since we do not know exactly what properties to ascribe to the theoretical model, we cannot make a strict comparison between model and muscle. PRYOR himself has concluded, apparently from a qualitative comparison between activation of his model and the "melting" of frozen rubber, that extensibility should be greater during activity than during rest: and he produces some evidence that this is the case also in living muscle. However, resting muscle is itself so highly extensible that one can hardly believe contracting muscle to be more

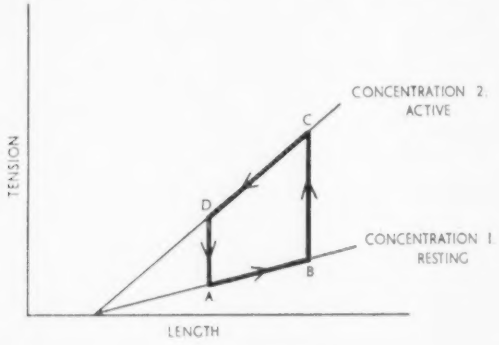

Fig. 23. See text

extensible still. Moreover, the oscillating stretch method which PRYOR used, bristles with difficulties of interpretation since it is quite wrong to describe contracting muscle as though it were a new elastic body (see p. 306 above).

It is to be hoped that PRYOR will develop his theory in greater detail, for there are many ways in which a chemical engine might work and some of the ways do not require that extensibility should be greater during activity than during rest. For example, if the modulus of an elastic body were altered by the plasticizer in the way shown in Fig. 23 the model could be taken round the cycle shown by the arrows and made to yield work proportional to the area *ABCD*. Note that this model is more extensible during rest than during activity.

Theory of Botts and Morales

The problem of muscular contraction naturally interests physical chemists. In general they approach the problem "from below," trying to discover what mechanisms at molecular level might account for contraction. One particular hypothesis has received a good deal of attention recently[62], [63], [64] and has been developed into a coherent theory of muscular contraction by BOTTS and MORALES.[61]

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It is suggested that in resting muscle the protein filaments are held in an extended configuration by electrostatic repulsion between positive ions attached to the filaments. When the muscle becomes active, negative ions of ATP become available to neutralize this charge; whereupon the filaments are freed to coil up under the influence of Brownian movement. Resting muscle can therefore be compared to rubber held in extension by electrostatic forces.

It is not at all clear what properties such a model will have at a macroscopic level, so one cannot easily tell whether the theory is, or is not, compatible with the known properties of muscle. Indeed, it is specifically stated that reloading mechanisms, whose properties are undefined, are in action at the same time as the electrostatic contractile mechanism. Such incompatabilities as can be predicted, e.g. that the model should absorb extra heat when it shortens, while muscle gives out extra heat, can then be explained as properties of the reloading mechanism.

By making assumptions about the number of protein filaments, the spacing of the resting positive charges etc., BOTTS and MORALES are able to calculate that the net electrostatic repulsive force is of the same order of magnitude as the measured maximum tension developed by a muscle. However, the calculation also indicated, as A. V. HILL has pointed out, that for a single twitch the model would need thirty times more ATP than can be demonstrated altogether in living muscle. Perhaps this discrepancy is not too serious, since one only expects agreement in orders of quantity; while MORALES suggests that one cannot necessarily identify the instantaneous *concentration* of ATP with the total mobilizable *amount* of it.

Other evidence for the validity of the theory is all of a somewhat indirect nature. It may be valid, but at present the theory is too vaguely stated to permit proof or disproof.

THE EVALUATION OF THEORIES

How should one judge the value of a scientific theory? Wrong theories and muddled thinking have lead to valuable advances often enough to encourage humility in a critic. Given the framework of classical physics, many a theory could be disposed of swiftly. But generally, and in biology specially, it is more difficult to erect strict criteria by which a theory is to stand or fall; often one is faced with a more difficult and less objective task. Small credit attaches to a very elaborate theory which can only interrelate a few facts; so in assessing the value of a theory one must somehow estimate, and then compare, both its complexity and its range of achievement.

Even in a simple case a large subjective element may enter. Imagine that we have some experimental results in the form of a graph (for

example, suppose that we have made measurements of the force exerted by a muscle at different shortening velocities, as in Fig. 14). Imagine too, that we also have a theoretical equation which we think should predict the shape of the force-velocity curve. In order to assess the accuracy with which theory fits experiment we have to consider that in the fitting process a number of arbitrary constants may have been used which had themselves been calculated from the experimental results. Theory and experiment are *obliged* to fit at a number of points equal to the number of constants calculated in this way. "Genuine" goodness of fit can thus be assessed only at other points on the curve. It would be good policy if the points of obligatory fit were indicated in some way in published evidence. As a rule we can only assess the "genuine" goodness of fit subjectively, for statistical methods can seldom be applied. Moreover, the chances are that several other equations could be found to give just as good a fit with the experimental curve; five equations at least fit the force-velocity curve. BERTRAND once said, "Give me three constants and I will draw an elephant: give me four and I will make him wave his trunk."

A similar process to the employment of many arbitrary constants is shown up in theories of a more qualitative kind when a new assumption is needed to dispose of each new difficulty. We all suffer from the temptation to elaborate our theories in this way, for few of us are strong-minded enough to follow BATESON's advice to "treasure our exceptions."

Even more suspect should be the theory which explains nothing because it can be adapted to explain anything. Such a theory can never be disproved, which gives it an illusory strength. Inviolable theories may be useful for some purposes (in politics perhaps) but a scientist must insist that theories be disprovable in principle, if not by an immediate experiment.

The evaluation of a particular theory may thus be a difficult and partly a subjective undertaking.

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